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**Clinical and microbiological aspects of periodontal disease in horses in  
South-East Queensland, Australia**

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## Abstract

The study of periodontal disease as part of equine dentistry is one of the overlooked fields of study, which truly needs more study and research to clearly understand the nature of the disease, the most appropriate diagnostic technique and prevention or treatment to provide for a good quality of life for horses.

The abattoir survey of the oral cavity and dentition of 400 horses from South-East Queensland, Australia, showed that the most common dental abnormality was sharp enamel points (55.3% prevalence). Several types of dental abnormalities were strongly associated with age. The highest frequency of dental abnormalities (97.5%) were observed in senior horses (11-15 years old) and this included periodontal disease that increased to almost fifty percent in senior horses. The findings also confirmed that all horses, not just young horses, should have regular complete dental examinations as early as possible which should limit the development of more severe dental pathologies later in life.

The equine oral microbiome found in dental plaque can cause oral disease which involves the some of the endogenous oral microbiota becoming opportunistic pathogens. The conventional method of oral microbiology based on culture dependent techniques usually overestimates the significance of species that are easily grown and overlooks microbial community diversity. Recently, the culture independent techniques using the next generation sequencing (NGS) method can determine the whole bacterial microbiota. The results from culture dependent and NGS method of analysis of healthy gingiva and periodontitis samples showed that the most common Genera isolated were *Prevotella* and *Porphyromonas* without a statistical significance in bacterial diversity, between the control and periodontitis groups. In conclusion, equine bacterial diversity between the healthy and periodontitis groups were similar which is different from other animals and this may be due to factors such as dentition, feed type and husbandry.

The standard approach for diagnosis of equine periodontal disease (EPD) is based on clinical dental examination but to evaluate the current stage of disease and for further treatment planning, auxiliary aids such radiography are required. Equine dental radiography has been largely restricted to extraoral techniques by the standard equine radiography technology and the complexity of equine head anatomy. In

addition, superimposition and distortion of the cheek teeth arcade is a major problem for radiographic interpretation. Intraoral radiography has been largely ignored in horses, due to the uncooperative nature of these patients and the absence of dedicated technology unsuitable for equine oral radiography. In this study, radiographic images from seven horse heads obtained using a commercially available equine intraoral computed radiography system (IO-CR) and 3D imaging from computed tomography file sets were reviewed. IO-CR has shown potential as a diagnostic tool where key pathological lesions of EPD of the cheek teeth were demonstrated without superimposition from the opposite dental arcade. 3D imaging is also another useful technique that has shown clear areas of alveolar bone loss and the ability to calculate accurate attachment lost where periodontal lesions are present. However cost and availability restricts the use of 3D imaging as a routine procedure. In conclusion, IO-CR provides good quality radiographs that could be used in diagnosis and treatment planning for equine periodontal diseases.

The first *in-vivo* study of the effect of a commercial preparation of 1.4% w/v chlorhexidine gluconate (CHX, Hexarinse®) on sub-gingival plaque using NGS method was studied. Samples were collected from the equine oral cavity before and after a three month CHX trial. The data of both pre- and post-trial oral plaque profiles were compared with no significant changes in the bacterial community observed. However a narrower range, but not statistically significant bacterial diversity was observed in the CHX group. In conclusion, 1.4% w/v CHX has an influence on the bacterial community by narrowing the diversity range and reducing the numbers of some periodontal bacteria. An important factor in its efficacy appears to be the duration, dosage and interval between applications. The long term effect of oral application of CHX needs to be studied as it is possible it could affect the intestinal microbiome and lead to other health concerns.

In conclusion, dental abnormalities such as sharp enamel points and periodontal diseases are common in horses where EPD has a higher prevalence in senior horses. EPD can be diagnosed easier using IO-CR with high quality images, whereas 3D imaging is more suitable for research and academic studies. Bacterial isolates from the equine oral cavity have a complex diversity with the most frequent isolates belonging to the genus *Bacteroides*. Currently there are limitations with the databases for molecular identification of oral bacteria from the equine oral cavity. In addition,

novel bacteria from the equine oral cavity are waiting to be discovered. The use of CHX as an oral rinse for horses has the potential for controlling and reducing the diversity of oral bacterial species but this was not shown to be significant in this study. The long term use and higher dose rates of CHX need further investigation due the effects of CHX on the intestinal microbiota.

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## Publications during candidature

### Peer-reviewed paper

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**List of Abbreviations used in the thesis**

2D	Two dimensional
3D	Three dimensional
AL	Alveolar bone loss
CB	Reduced crestal bone height
CHX	Chlorhexidine gluconate
CI	Confidence interval
CLM	Clindamycin
CR	Compute radiography
CT	Computed tomography
CXT	Chlorhexidine treatment group
DHL	Doxycycline hydrochloride
DI	Diastema
DICOM	Digital imaging and communications in medicine
DR	Digital radiography
EO	Extraoral open mouth
EOTRH	Equine odontoclastic tooth resorption and hypercementosis
EPD	Equine periodontal disease
ETR	Exaggerated transverse ridges
FL	Inter radicular bone loss/furcation lesion
IO	Intraoral
IO-CR	Intraoral compute radiography

MRI	Magnetic resonance imaging
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PCA	Principal component analysis
PD	Periodontal disease
PDL	Periodontal ligament
PP	Periodontal pocket
SEP	Sharp enamel points
WCA	Wilkins Chalgren agar
WCB	Wilkins Chalgren broth
WF	Wet film preparation
WS	Widening of the periodontal ligament space
Wt	Water treatment group

# **Chapter 1:**

## **Introduction**

Periodontal diseases (PD) are groups of diseases that affect one or more of the periodontal structures i.e. tooth, alveolar bone, periodontal ligament, and gingiva. PD can be divided into two stages: gingivitis and periodontitis. Gingivitis is a reversible inflammation of the gingiva due to accumulation of plaque, whereas periodontitis is an irreversible inflammation that involves loss of the supporting structures of the teeth. Gingivitis can be easily visualized by normal examination, appearing as swelling and reddening of the gingiva. By contrast, in periodontitis inflammation and subsequent attachment loss in the deeper periodontal tissues is more difficult to assess.

Equine periodontal disease (EPD) had been recognised for many years as a major problem (Scott & Tony 2002). The majority of horses show some evidence of PD, and the incidence is up to 60% in senior horses (Baker 1979). Although Australia's horse population is estimated to be around 1.2 million (Gordon 2001), only one large scale surveys investigating equine general dental health have been conducted in Australia (McGowan et al. 2010). Little is known about the prevalence of dental disease in the Australian horse population. Improved knowledge of the prevalence of dental disease would raise awareness in the equine veterinary profession of this underlying health problem (Chapter 3).

Unlike other domestic animals, horses have two different types of teeth: the brachydont teeth (canines and wolf teeth) and the hypsodont teeth (incisors and cheek teeth). Generally, horses do not appear to have significant problems with periodontal disease in erupting permanent brachydont teeth (Dixon et al. 2000c), although it has been reported in association with equine odontoclastic tooth resorptive and hypercementosis (Staszyk et al. 2008; Lee 2010; Chinkangsadarn et al. 2013). Primary EPD is more common in the hypsodont cheek teeth; up to one third of the population in one study were affected (Baker 1970).

The pathophysiology of EPD starts with changes in the oral bacterial population, from Gram-positive aerobic bacteria to Gram-negative anaerobic bacteria (Marsh 2003). This causes an inflammatory response within the periodontium. Initially, apical detachment of junctional epithelium forms a periodontal pocket and bacteria accumulate in the gingival sulcus, with clinical signs of redness, bleeding and swelling of the gingivae resulting in gingivitis. The host response to the sub-gingival bacteria

involves osteoclastic activity that causes loss of supporting alveolar bone and eventually leads to loss of the tooth.

The dental plaque bacteria in animals with PD has been studied in the dogs (Yamasaki et al. 2012; Riggio et al. 2011), cats (Girard et al. 2009; Booij-Vrieling et al. 2010), sheep (Spence et al. 1980; McCourtie 1990), donkeys (Takada et al. 2010), marsupials (Bird et al. 2002) and horses (Bailey & Love 1991; Racklyeft & Love 2000; Dorsch et al. 2001). However, there are no reports of equine dental plaque bacteria that compare healthy and gingivitis or periodontitis-affected horses. Equine oral bacterial plaque ecology studies may provide the basic knowledge on how EPD occurs and how it progresses (Chapter 4).

EPD diagnosis is usually based on visual clinical examination and palpation during an oral examination, and some veterinarians may use endoscopy and radiography for better visualisation (Simhofer et al. 2008). Equine head and dental radiography was introduced many years ago (Gibbs 1974), but more recent advances in radiography, including computed radiography (CR) and digital radiography (DR), have increased the sensitivity and specificity of dental pathology detection (Townsend et al. 2011). Extraoral dental radiography is the most common imaging technique used in horses, but the horse skull has many complex structures which restrict the beam angle necessary for achieving a good diagnostic image and cause superimposition of dental arcades and distortion of the tooth of interest. Equine dental radiography can be improved by isolating the dental arcade using intraoral techniques and use of the bisecting angle technique. The intraoral technique has been previously described with differing methodologies ranging from using cut wet films to modified CR plates (O'Brien 1996; Klugh 2005f; Uhlhorn et al. 2008), but these methods may affect the image quality and patient tolerance. A new innovation involving custom made equine dental intraoral computed radiography (IO-CR) promises to bring dental radiography to the next level in radiographic imaging. Research quality computed tomography (CT) equipment can provide images with 0.5 mm slice thickness, with the additional advantage of allowing three dimensional (3D) image reconstruction from the CT dataset. This produces excellent anatomical imaging that is not possible through two dimensional (2D) images. The 3D technique may be the way forward for equine veterinarians to acquire a precise diagnosis of dental disease and for optimal planning of EPD treatment or to establish new surgical procedures. (Chapter 5).



The microbiota of periodontal plaque plays an important role in the pathogenesis of EPD. Controlling pathological bacteria that initiate the disease is the goal for disease prevention and control. Chlorhexidine gluconate has a long history of safe and effective use in plaque control in humans (Rindom Schiøtt et al. 1970; Briner et al. 1986; Sreenivasan & Gittins 2004; Tomas et al. 2008) but the bitter taste precludes it for daily use in horses. Studying the effect of non-bitter chlorhexidine gluconate (CHX) mouth rinse on horse bacterial plaque communities using next generation sequencing (NGS) could potentially show whether EPD can be easily prevented and possibility controlled (Chapter 6).

The primary hypothesis of this thesis is that equine periodontal disease is one of the common dental abnormalities in Australian horses, and it can be diagnosed radiographically using intraoral radiography. A second hypothesis is that in periodontally healthy horses, Gram-positive microbiota predominate and in horses with periodontitis, Gram negative anaerobic bacteria predominate. Finally, this thesis hypothesises that Gram-negative, anaerobic, periodontopathic bacteria can be controlled with daily use of chlorhexidine gluconate, and disease progression will be subsequently reduced.

The objectives of this investigation were to:

Determine the prevalence of dental disorders in horses in South-East Queensland, Australia.

Identify bacteria from subgingival plaque in horses using culture dependent methods and culture independent molecular methods, and determine differences in bacterial communities between healthy and periodontitis affected horses.

Review the use of IO-CR and 3D imaging from computed tomography image sets as aids in EPD diagnostics.

Determine the effect of chlorhexidine gluconate mouth rinse on equine oral bacteria to determine whether a daily treatment protocol will control periodontal disease in horses.

## **Chapter 2:**

### **Equine periodontal disease; A review of the literature**

## **Equine dental anatomy**

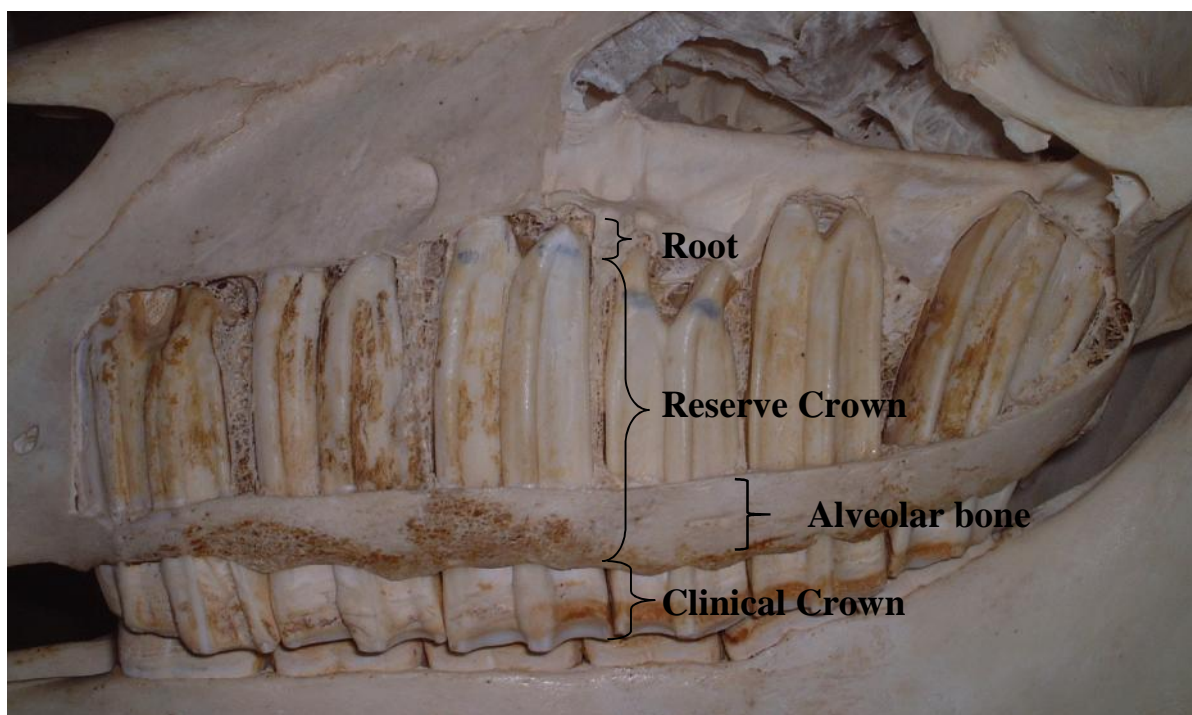
Horses belong to the family Equidae. Equine canine and first premolar teeth are short crowned and known as brachydont teeth, but most equine teeth are referred to as “hypsodont teeth”; they have long-crowns that continually erupt, wear and develop roots later in life. The hypsodont tooth crown consists of two parts, the visible or exposed clinical crown that can be seen above the gum margin and the reserve crown that is buried deep under the gum in the alveolar bone. In hypsodont teeth, the root refers to the apical area of tooth without enamel and unidentifiable crown-root junction as shown in Figure 1. A hypsodont tooth consists of three elements:

1. The outer layer is cementum which covers the whole crown and root (root cement) (Mitchell et al. 2003), unlike brachydont teeth, which have enamel as the outer layer of the crown and cementum only covers the root surface. The main function of cementum is to connect the tooth with surrounding structures via the periodontal ligament (PDL) and ensure that tooth is strongly embedded.

2. The layer beneath the cementum is enamel, which is considered the hardest substance in the body.

3. The core structure of the tooth is the cream coloured substance called “dentine”, which constitutes a major part of tooth. Dentine itself is not as hard as enamel and can be darker in colour due to the absorption of food pigment into the dentinal tubules. In incisors and maxillary cheek teeth, enamel infolding creates the infundibulum which is partially filled with cementum. In young horse incisors the infundibulum is large (called the “dental cup”), which becomes smaller and shallower from feed grinding over time. Eventually, the remaining “dental star” is actually secondary dentine that is formed to protect the pulp chamber as the tooth wears down.

The horse’s maxilla is wider than the mandible (“anisognathia”). This creates a discrepancy in the cheek teeth position, which combined with normal mastication creates a 10-15% slope on the occlusal surface of the cheek teeth arcades (Brown et al. 2008; Dixon & Dacre 2005; Hillson 2005; Wilson 2006; Dixon & du Toit 2011).



**Figure 1** The maxillary cheek teeth of a four year old horse show long reserve crowns with short clinical crowns and developing roots.

### Dental formulae

A number of alternative nomenclature have been proposed to describe equine teeth, such as the anatomical system, the cheek teeth system and the modified Triadan system. The modified Triadan system is composed of three digits, the first digit represents the quadrant of the tooth arcade starting from the upper right of the animal and moving clockwise; the last two digits represent the number of the tooth in each arcade from the central incisor to the last (third) molar (Floyd 1991). The dental formulation of the deciduous and permanent teeth of the horse is described below using the Triadan system.

Deciduous teeth: 2 (3/3, 0/0, 3/3) = total 24 teeth.

Permanent teeth: 2 (3/3, 1/1 or 0/0, 3/3 or 4/4, 3/3) = total 36 to 44 teeth depend on the presence of the canine and the first premolar teeth or “wolf teeth” (Hillson 2005).

## Incisors

Equine permanent incisor teeth wear down and erupt throughout life, producing age-related changes that can be used to estimate the animal's age (Galvayne 1886). The adult horse has 12 incisor teeth with tight alignment to each other; however, with increasing age the space between them becomes wider due to wear and the eruption process of the narrower reserve crown and root.

## Canine

The canines or "tushes" are brachydont teeth in the horse; these teeth are fully erupted at four to five years of age, after which they stop growing. Male horses normally have two upper maxillary and two lower mandibular canine teeth, which are usually absent in females or if present are only small. The canine teeth have no function in the eating or chewing process, and are used for biting and fighting during the male's offensive behaviour. Sometimes they can be sharp and need to be reduced or blunted for protection of humans and other animals or even themselves (Easley 2005).

## Cheek teeth

The horse cheek teeth are comprised of molar and permanent premolar teeth. The first premolar teeth, sometimes called "wolf teeth", are small brachydont teeth and occasionally present in some horses with visible crown or unerupted crown. However, the wolf teeth perform no function and can cause discomfort and pain when they are in contact with a bit. For this reason, they are often extracted. The set of premolar and molar teeth (second premolar to third molar) function as a single unit.

Using dentition to estimate a horse's age has been part of horsemanship for a long period of time (Galvayne 1886). The accuracy of this age approximation is influenced by a number of factors that affect wear and eruption patterns, such type of feed, behaviour and other environmental factors.

The determination of age in the horse is based on the following changes:

The eruption times of permanent and deciduous incisors.

The shape and appearance of the occlusal surface of mandibular incisors.

The bite alignment of incisor arcades.

The occurrence of anatomical changes, such as hooks and grooves on the upper corner incisors.

The eruption times of the permanent molars and premolars.

The accuracy of age determining by dentition will decrease after all of the permanent incisors are in full contact at 6 years old (Wilson 2006). Then it will be based on the wear pattern of incisors from attrition forces and other anatomic changes.

### **Anatomy of equine periodontium**

Anatomically, the periodontium consists of the gingiva, the periodontal ligament (PDL), the alveolar bone, and the root cementum. The primary function of the periodontium is to support the teeth and maintain them in the alveolar bone.

With increasing age, these supporting structures can undergo changes due to inflammation, environmental changes, such as, type of diets, feeding behaviour and altered morphology due to excessive wear of teeth (Lindhe & Lello 2009).

The periodontium consists of specialised tissues that surround and support the teeth. The gingiva can be divided into marginal gingiva (the collar-like margin of gingiva surrounding the tooth) and attached gingiva (next to the marginal gingiva and securely attached to the periosteum and alveolar bone, extending to the mucogingival junction). The marginal gingival margin, which is not attached to tooth cementum, creates a space called the gingival sulcus. Microscopically, gingival epithelium is divided by location. The junctional epithelium lies at the apical aspect of the gingival sulcus, which is lined by sulcular epithelium. The outer epithelium covers the gingival margin and oral surface of marginal and attached gingiva. The regeneration process of these epithelial cells initially starts from the basal layer of the junctional epithelium. While the dividing cells migrate occlusally and centrifugally, the reattachment process continues on at the basal lamina of the tooth (Klugh 2005a). The interdental papilla of equine teeth is bucco-lingually wider with closer contact between adjacent teeth than brachydont teeth, making it easier to damage the periodontium (Klugh 2005). The hypsodont teeth of the horse have a cementum layer covering the entire tooth after eruption. This wears away from occlusal contact and exposes the enamel and dentine.

Therefore these teeth have no cemento-enamel junction, which is the common attachment site of the PDL in brachydont teeth. Sahara (2014) concluded that the coronal cementum of horse cheek teeth is a multistructural and multifunctional tissue and is a major structural component of the occlusal surface area allowing distribution of forces from mastication and helps prevent enamel fracture. In addition, the study of tensile stress of equine cheek teeth periodontal ligament using finite element (FE) on 3D models showed an increase in intrusion with increasing age due to age-related changes in the periodontium but well within suggested periodontal ligament ultimate tensile stress (Cordes et al. 2012).

Recently specific terminology has been proposed to describe equine PDL fibres based on the orientation of the fibres and their attachment to anatomical structures: cementogingival, periostealgingival, cementoperiosteal, alveocemental, cementoperiodontal, cementocemental, alveoloalveolar, periodontoperiodontal, gingivogingival (Staszyk et al. 2006).

### **Eruption of the teeth**

Tooth eruption is the developmental movement of the tooth from its osseous crypt position into oral cavity until its contact with the opposite tooth. The mechanisms of prolonged eruption of equine hypsodont teeth remain unclear (Baker 2005b). A number of theories have been proposed to explain the major drivers of hypsodont tooth eruption: a) growth of the tooth root, b) hydrostatic vascular pressure, c) alveolar bone resorption and formation and d) mechanical retraction of the PDL. Of these, the most likely mechanism playing the major role in equine hypsodont tooth eruption is osteoclastogenesis and osteogenesis of alveolar bone (Wise & King 2008). While the others mechanisms are probably invalid due to supported studies such as: 1) the eruption of tooth can occur without root and crown (Gowgiel 1961; Marks Jr & Cahill 1984); 2) the intra-osseous phase of eruption process started before the attachment of the periodontal ligament to cementum and alveolar bone (Cahill & Marks 1982; Marks Jr & Cahill 1984; Wise et al. 2007); 3) uncertain and conflicting of results in the study of the effect of vascular pressure on tooth eruption (Cheek et al. 2002; Shimada et al. 2004).

### **Continuous reattachment of equine periodontium**

The hypsodont teeth of horses have a unique periodontal anatomy with continuously erupt throughout a long period of their life (up to 9mm/year), unlike brachydont teeth that erupt just to the occlusal point without further eruption (Kirkland et al. 1996; Baker 2005a). This is a useful adaptation to the massive masticatory process (approximately 18 hour per day) necessary for the herbivore diet (Staszyk & Gasse 2007). In order to compensate for normal rapid attrition of the occlusal surface, continuous eruption of reserve crown occurs, which varies from 3 mm. to 9 mm. per year, depending on the conditions of the horse (Kirkland et al. 1996; White & Dixon 2010).

The continuous eruption of teeth involves the surrounding tooth structures, including the PDL, cementum, alveolar bone, etc., which also need to be continuously reattached and remodelled. According to Staszyk and others, equine periodontal structures have three functions: 1) to fix the tooth in the alveolus; 2) to support the tooth during the mastication process and; 3) to restore the tooth to its original position (Staszyk and Gasse 2005).

The equine PDL is the continuous connective tissue that extends from gingival to tooth cementum at attached gingival area with major fibres called principal fibre with collagen fibres called “Sharpey’s fibre” are securely embedded in the dental cementum or alveolar bone (Mitchell et al. 2003; Warhonowicz et al. 2006). More than half of PDL’s structures are composed of various types collagen fibres mixed with a small number of blood vessels and nerve endings (Fill et al. 2011). The elastic fibre system in the PDL is mainly composed of the independent fibres called “Oxytalan fibres” which can be found in two well-defined groups, the blood vessel related fibres and the independent fibres (Staszyk & Gasse 2004a). In comparison, the equine PDL also has more elastic fibres than brachydont PDL (Klugh 2005a). The oxytalan fibres have a function to prevent the dislocation of the vascular system during the mastication process (Chantawiboonchai et al. 1999). Surprisingly, the young equine tooth is partially attached by periodontal ligaments, with attachments absent towards the apex of the tooth, and present at the gingival level and the occlusal aspect of the reserve crown. Then, the maturation attachment of PDL’s suspensory apparatus will progress apically as the tooth ages (Staszyk et al. 2006).



## **Epidemiology of equine dental abnormality**

Dental abnormalities and oral diseases in horses are the underlying cause of many general disorders, such as weight loss, pain, behavioural abnormalities, poor performance. The most common dental abnormality in the horse is over growth of enamel points, which develop on the buccal edge of maxillary cheek teeth and the lingual edge of mandibular teeth leading to ulceration of surrounding soft tissues such as buccal mucosa and tongue causing discomfort to the horse (Brigham & Duncanson 2000a; Simhofer et al. 2008; Anthony et al. 2010). In order to perform a thorough clinical examination for dental disorders and abnormalities, the horse needs to be sedated and examined using a full mouth speculum. The dental examination should include an external examination of the skull and surrounding soft tissues and evaluation of head symmetry.

### **Disorders of incisor, canine and first premolar (wolf) teeth**

Disorders in these teeth are not common compared to disorders of the cheek teeth, only 14% of referred cases reported in one study were from primary non-cheek teeth (Dixon et al. 1999a). Disorders of incisors included retained deciduous teeth, supernumerary teeth, abnormalities of wear and malocclusion. The majority of abnormalities were secondary to cheek teeth disorders. However, increasing awareness among equine veterinarians of the importance of a thorough oral examination is only a recent development, and poor awareness may have biased the reported results.

Equine odontoclastic tooth resorption and hypercementosis is a painful disorder seen only in incisors and canines and usually has some degree of complication with periodontal disease (Staszuk et al. 2008). Yet, the aetiology of this disorder is still unknown. The first premolar or wolf tooth is a non-chewing tooth and is usually extracted in working horses, as this tooth may interfere with bit placement. Some disorders of the first premolar are pulp necrosis from fracture or exposed pulp and blind or unerupted wolf tooth (Dixon & Dacre 2005).

## **Disorders of cheek teeth**

Almost 90% of referred dental related cases at the Edinburgh Veterinary School were for treatment of cheek teeth disorders (Dixon et al. 1999b). The most common cheek teeth abnormalities diagnosed included the following:

Disorders of development and eruption, such as a retained deciduous tooth (cap), malposition and displacement of a tooth, a supernumerary tooth, and eruption cysts.

Disorders of wear such as sharp enamel point (SEP), hook, ramp, stepped tooth, of pathologies usually result from overgrowth of teeth, loss of teeth and displacement of teeth.

Traumatic disorders or fractured teeth, mostly caused by kicking or biting and some resulting from dental treatments. The usual presenting signs of trauma are quidding and the presence of external sinus tracks through the cheeks or facial crest due to infection (Dixon et al. 2000a).

Apical infection of cheek teeth: the common signs are external swelling, external sinus tracks and quidding. Many studies found that the most commonly affected teeth were third and fourth mandibular premolars and fourth maxillary premolars, with average age at onset of clinical signs of five to seven years old. (Baker 1970; Lane 1994; Dixon et al. 2000a).

Periodontal disease: this abnormality involves the destruction of periodontium, and is considered the most significant problem among horse dental disorders. The early sign of periodontal disease in horses can be recognised by reddening of the gingival margin of incisors and canines. If untreated or not controlled, this lesion may progress to involve destruction of the periodontium, "periodontitis", with lesions such as diastema (DI, a gap at the interdental space), periodontal pockets and mobile tooth. However, the reported high incidence of this disease could be anomalous due to misdiagnosis or being secondary to other periodontal symptoms such as DI or tooth displacement (Dixon & Dacre 2005).

## **Periodontal disease**

Periodontal disease (PD) is one of the most widespread diseases and has been reported in a number of animal species and humans. There are many different types of dentition, such as hypsodont, oligodont or brachydont, and each type of dentition has a unique anatomic structure of periodontium.

### **Periodontal disease in dogs and cats**

PD is common in dogs and cats with a prevalence as high as 80-85%. One study showed more than 70 percent of dogs and cats under the age of two years had some form of PD, with the incidence rising with smaller dog breeds and as the animals increase in age (Watson 1994; Hoffmann & Gaengler 1996; Niemiec 2008). In the review study of Watson (1994), dogs that were fed with dry food had less periodontal related problems than those fed with soft food, possibly due to the abrasive action of the dry food helping to reduce plaque formation.

In a large computerized retrospective data analysis study among privately owned veterinary hospitals in U.S., there was a positive relationship between PD and clinical diagnosis of azotaemic canine kidney disease, and the severity of PD increased with increasing age and body weight. The relationship between PD and high body weight may have been due to dogs being fed soft texture food (Glickman et al. 2011). In similar study on dietary change from soft food to raw meaty bone food with ongoing periodontal disease treatment in leucopenic dogs and cats, the result shown that the red and white blood cell count has increase in treated animals (Lonsdale 1995). In another study, the relationship between PD in dogs and the risk of other common diseases (such as cardiovascular disease, cruciate ligament rupture, urinary incontinence, aggressive behaviour) was studied. A strong association was found between the severity of PD and the risk of endocardiac disease (up to six times higher in dogs with PD grade three compared to non-PD and PD grade one), but no relationships were found between in the risk of other common diseases and the degree of PD (Glickman et al. 2009).

### **Periodontal disease in rabbits and rodents**

Rabbits and rodents with natural diets consisting of grass and hay have a low incidence of PD due to the anatomy of their full elodont teeth (open-rooted teeth) with continuous growth (Lennox 2008) of incisors and cheek teeth (premolar and molar), which minimizes the accumulation of food debris (Verstraete 2003; Crossley & Aiken 2004).

### **Periodontal disease in stock animals**

PD in swine is usually associated with poorly performed malpractice teeth clipping techniques, resulting in gingival damage (Straw et al. 2006). Unlike from other domestic animals, sheep PD are affect incisors called “broken mouth” or premature incisor loss which caused while others are suffering from cheek tooth (McCourtie 1990; Spence et al. 1980). The abattoir survey on hill sheep found that 17% of the collected samples had loose teeth with almost half of the samples had missing teeth. The highest incident of missing teeth were incisors followed by premolars and molars (Aitchison & Spence 1984). PD in sheep are also know to affected their productivity and culling of sheep that unable maintain good body condition is the common practice among the farmer (McGregor 2011; Navarre et al. 2002; Ridler & West 2010). In contrast, dental problems in cattle which share the same type of hypsodont teeth (the high-crowned teeth with continuous eruption (Dixon & du Toit 2011)) with sheep are not commonly a major cause of clinical diseases. Surprisingly, industrial and urban type cattle farms tend to have more periodontal related abnormalities than cattle raised in small farms. This may due to difference in feeding practice by farmers. One common periodontal related disorder in cattle is “swollen face” disease or alveolar periostitis which is severe periodontitis with secondary bacterial infection. The clinical sign of bilateral swelling of the cheeks is caused by feed/grass trapping between teeth creating periodontal pockets (PP) resulting in bacterial infection. This oral disease may lead to malnutrition or even death (Tuns 2010).

### **Periodontal disease in wild animals**

The study of dental abnormalities in free-ranging adult and yearling shot reindeer in the sub-Antarctic island of South Georgia during the mid-1900s found a high prevalence of molar damage and mandibular swelling (lumpy jaw) among three herds of shot reindeers (Leader-Williams 1980). This differs from other studies that show a

lower incidence of dental diseases (Leader-Williams 1980). There were many dentistry related studies in free-ranging non-human primates that have low-crowned teeth called brachydont teeth such as, Baboons, Orang-utan and Ring-tailed lemur. The comparative research study between size of second molars in captive research baboons and wild baboons for determining the genetic relationship between them revealed that the captive baboons share the phenotype with the wild baboons (Hlusko & Mahaney 2007). Studies in dental pathology, related feeding behaviour of Orang-utan and Ring-tailed Lemurs and showed that there were associations between feeding behaviour and type of feed. Local ecological factors and feeding behaviour, differences between male and female monkeys affected the prevalence of PD and loss of teeth (Hall et al. 1967, Stoner 1995). The study of Swedish brown bears illustrated that all of them had incomplete dentition from un-erupted teeth in juvenile bears, missing one or more teeth. Surprisingly none of these bears had evidence of caries which was considered a source of PD. However, this study is limited due to the short time allowance under general anaesthesia. This gave only rough estimates of whole mouth periodontal index, while individual teeth examinations are more accurate for clear understanding in determining the stage of PD in animals (Strömquist et al. 2009).

From the latter studies in wild animals, the occurrence of PD relate to risk factors such as the age of animal, type of diet and feeding behaviour. The anatomic structure of teeth and periodontium are the same between captive and wild animals. Therefore in a comparative study of free-ranging and stabled horses it is differences in risk factors that are important and not the structure of the periodontium and teeth.

### **Periodontal disease in non-equine equidae**

The reported studies of dental diseases in donkeys are limited and species-specific information not available and therefore based on the equine dental literature. Even though donkeys and horses are from the same family, Equidae they do not have identical dental anatomy. The study of post mortem donkey skulls in United Kingdom has shown that donkeys have greater degree of anisognathia (wilder width of maxillary than mandibular arcades) and greater degree of enamel infolding of their mandibular than maxillary arcades compared to horses (Du Toit et al. 2008a, 2008b). Moreover, the study of dental disease in Mexican donkeys, used as transport animals, found that

up to 60% of the population had some type of dental disease and most suffered from sharp enamel points. Dental disorders associated with periodontal disease include overgrown, worn teeth, missing teeth, displaced teeth and malocclusion of the dental arcade were commonly found in the higher aged group (over 21 years old) (Dutoit et al. 2008).

### **Aetiology of periodontal disease**

The oral microbiota is comprised of many microorganisms, such as bacteria, fungi, mycoplasma, protozoa, and viruses. Currently the aetiology of periodontal disease is thought to comprise three main factors: a susceptible host, the presence of pathogenic species or a keystone pathogen that will foster the growth of other bacterial species or at least not inhibit their growth and time (Socransky & Haffajee 1992; Hajishengallis et al. 2012). In earlier oral microbiology studies, two early controversial theories to explain bacterial plaque formation were proposed: the specific plaque hypothesis suggested that PD is caused by overgrowth of certain indigenous bacteria (Loesche 1979) whereas the nonspecific plaque hypothesis proposed that different combinations of indigenous bacteria are responsible for progression of disease from gingivitis to destructive periodontitis (Theilade 1986). Recently, another theory, the ecological plaque hypothesis proposed that specific periodontal pathogens do not initiate periodontitis in isolation, but different members of the microbial community operate synergistically (Marsh 2003). This hypothesis proposed that the effects of environmental and bacterial factors changed the oral environment to a more anaerobic one suitable to promote a shift in the bacterial ecological species of indigenous periodontopathic bacteria found in the subgingival plaque as opportunistic pathogens and can be found also in non-diseased areas. According to the ecological plaque hypothesis, correcting the environmental factors would be more effective in arresting PD than targeting periodontopathic bacteria directly. PD was initiated by a shift in bacterial populations from Gram-positive cocci and rods to an increased number of Gram-negative anaerobic rods, and in more severe periodontitis, motile spiral bacteria and spirochaetes were present (Marsh 1994).

The most current hypothesis is “the polymicrobial synergy and disbiosis model”, which hypothesised that periodontitis was initiated by the synergy between different members of the microbial community, which created a disease-provoking microbiota, rather than specific individual pathogens (Hajishengallis & Lamont 2012). The same authors have also proposed “The key stone-pathogen hypothesis”, which stated that periodontal disease is an infectious disease and not an opportunistic one which is initiated by low-abundance microbial pathogens, such *P. gingivalis*, which induced inflammatory disease by remodelling a normally benign symbiotic microbiota into a dysbiotic one (Hajishengallis et al. 2012). However, these new theories have only been proposed for humans. Further investigations of the equine oral microbiota and its association with disease status will be needed to confirm whether keystone bacteria are the same or different in human and equine periodontal disease.

### **The microbiology of equine periodontal disease**

Studies of equine oral microbiology have lagged behind those of human studies (Moore & Moore 1994; Aas et al. 2005) and that of other animal species (discussed above). An early study of the oral bacteria from the mouth and nose of normal horses found that most bacteria were Gram positive, spore bearing bacteria (Boyer, 1918). In their conclusions, the normal horse harboured a large “flora” of micro-organisms that were mostly harmless while a few may become pathogenic under certain conditions (Boyer, 1918). This conclusion is relatively unchanged to the present day, whereas now, there is an increase in diversity of bacteria, improvements in identification of the bacterial species and associations of these bacteria to oral and other diseases is now possible. Bacteria residing in the oral cavity was the source of many of the reported respiratory tract infections in horses (Bailey and Love, 1991). The respiratory infections had been of interest since mid-1800, when Falke published his treatise on the common respiratory diseases encountered in horses (Edwards, 1935). A number of studies were conducted during the 1980s, cited in Bailey and Love (1991), investigating the oral associated bacterial infections in horses from dental abscesses and respiratory infections. Strong evidence reported by Bailey and Love (1991) for direct associations between organisms present in the equine pharynx and respiratory diseases and supported by the work of Finegold (1977), showed that aspiration is a predisposing factor, especially in equine diseases (Mansmann, 1983). This work leads on to the requirement for identification of bacteria found in the oral cavity of horses.

Here, Bailey and Love (1991) identified 270 bacterial isolates from 12 horses, cultivated from the pharyngeal tonsillar surface and associated with lower respiratory tract infections showed that 98 isolates were obligate anaerobes belonging to seven genera: *Peptostreptococcus*, *Eubacterium*, *Clostridium*, *Veillonella*, *Megasphaera*, *Bacteroides* and *Fusobacterium*.

Mackintosh and Colles (1987) in a case study reported cultivation of mixed obligate, anaerobic bacteria belonging to *Bacteroides*, *Fusobacterium* and *Peptostreptococcus* species from a dental abscess in a horse and a donkey. All bacteria were sensitive to metronidazole and penicillin, except *Bacteroides fragilis* which was resistant to penicillin. The significance of the isolations is important and required antibiotic treatment. The long term (6 to 8 weeks) combination with trimethoprim- sulphonamide and metronidazole antimicrobial therapy is recommended in early primary apical and periapical abcessation in horses (Gerard 2006). However, if the recurrent clinical signs are present, tooth extraction is the most common definitive approach (Gayle et al. 1999, Boutros 2001).

Black pigmented, anaerobic bacteria belonging to *Porphyromonas* sp., (previously *Bacteroides*) are major periodontopathogens. In human periodontal disease, *P. gingivalis* has the ability to attach to motile bacteria, such as *Fusobacterium nucleatum* and can be translocated (Grenier 2013) and invade oral epithelia *in-vitro* (Sandros et al. 1993, Sandros et al. 1994). Other commonly detected black pigmented anaerobes found in subgingival plaque are the *Prevotella* spp., (previously *Bacteroides melaninogenicus*) (Shah & Collins 1990) and the human periodontopathic species being *Prevotella intermedia* (Socransky & Haffajee 1992; Moore & Moore 1994). Recently, Takada (2010) proposed a novel *Prevotella* species isolated from the donkey oral cavity named *Prevotella dentasini*. As with all 22 *Prevotella* species, their role in periodontal disease especially that of EPD requires further study.

*Fusobacterium* spp., another of the Gram negative anaerobic bacteria, are non-sporing fusiform/spindle shaped rods, some of which are part of the oral bacterial community, as well as being involved in soft-tissue infections in humans and animals (Dorsch et al. 2001). Oral fusobacterial species are intermediate colonizers within the biofilm that bridges the attachment of commensal bacteria to other pathogens



(Kolenbrander 2000). *F. nucleatum*, is an important human pathogen of periodontal disease (Citron, 2002), due to its ability to congregate and form mutual synergisms with other bacterial pathogens (Hajishengallis & Lamont 2012). *F. nucleatum* has been associated with periodontal disease (March 1994) and has been isolated from many animal species including dogs (Syed et al. 1981), cats (Mallonee et al. 1988) and horses (Bailey & Love 1991). *F. nucleatum* is also part of a small number of oral species that are associated with periodontitis, one of the most common infections in humans and animals. However, *F. nucleatum* was recently reported not to be directly responsible for destructive periodontal disease, which is a major cause of tooth loss (Signat et al. 2011). A new species, *Fusobacterium equinum* was isolated from the normal oral cavity and oral associated disease of horses. A number of the isolates were phenotypically and phylogenetically close to *Fusobacterium necrophorum* but were distinct from it however clustered with *F. necrophorum* within the *Fusobacterium* genera (Dorsch et al. 2001).

Another group of the equine periodontal-related bacteria are the spirochaetes and these were reported in a histopathology study of 22 horse skulls assessed for the presence of EPD. Four of the skulls showed the presence of spirochaetes using Gram stain and silver impregnation stain. This was the first time spirochaetes were shown to be associated with EPD. (Cox et al. 2012). Sykora (2014) reported the isolation of spirochaetes, when DNA purified from crevicular fluid derived from 23 horses affected with equine odontoclastic tooth resorption and hypercementosis (EOTRH) horses and 21 disease-free horses was tested for the presence of *Treponema* spp., *Tannerella* spp. and *P. gingivalis* by polymerase chain reaction. Subsequently, amplified DNA was bidirectionally sequenced and identified via BLAST analysis.

This study was based on the hypothesis that red complex bacteria, i.e. *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia*, are involved in the onset and progression of periodontal disease in humans, yet seldom inhabit the oral cavity of healthy individuals (Socransky et al. 1998). The study showed that *Treponema* and/or *Tannerella* DNA was detected in 100% of periodontitis-related samples and in 52.2% of DNA derived from healthy horses. Twenty-six amplicon sequences were 98-100% homologous to published bacterial sequences, which mostly corresponded to *Treponema pectinovorum*, oral *Treponema* clones JU025 and OMZ 840. *Tannerella*

*forsythia* and *P. gingivalis* DNA was only found in 3 EOTRH-related samples. Forty-three amplicon sequences revealed weaker homologies ranging between 80% and 97% to known *Treponema* or *Tannerella* strains, partly because of their heterogeneity, partly because they obviously represented so far unknown types *Treponema* and a *Tannerella* spp. They concluded that a novel *Treponema* and *Tannerella* spp. were isolated in association with EOTRH-related periodontal disease. However, *P. gingivalis* is a human pathogen which was only identified in three horses in this study where the animal *Porphyromonas* species is “*Porphyromonas gulae*” (Fournier et al. 2001) or maybe an “unknown equine *Porphyromonas* spp.,” which may be found in horses.

Another novel species, a Gram positive bacterium was isolated from the equidae family from two caries lesions, *Streptococcus* spp., *Streptococcus orisasini*, *Streptococcus dentasini* (Takada et al. 2010).

A recent study on the equine oral microbiome showed that bacterial taxa from 12 phyla from a 16S rRNA gene amplicon pyrosequencing of 200 pooled subgingival sites from two horses identified, that Proteobacteria (37.67%), Firmicutes (27.57%) and Bacteroidetes (25.11%) were the dominant phyla comprising over 90% of the total read from two samples (Gao et al. 2015). The other minor phyla identified showed Actinobacteria (3.17%), Chloroflexi (0.04%), Fusobacteria (5.15%), Spirochaetes (0.15%), Synergistetes (0.22%), Tenericutes (0.16%), GN02 (0.19%), SR1 (0.01%) and TM7 (0.37%). Many OTUs were not closely related to known phylotypes, and may represent ‘equine-specific’ taxa. Phylotypes corresponding to Gammaproteobacteria were abundant, including *Actinobacillus* spp. (8.75%), unclassified Pasteurellaceae (9.90%) and *Moraxella* spp. (9.58%) (Gao et al. 2015).

### **Bacterial culture and identification;**

There are two main methods of identification of bacterial species: culture dependent and non-culture dependent or molecular methods. The culture dependent method relies on the growth of bacteria in a suitable medium. Since most oral bacteria are facultative anaerobes or obligate anaerobes, a special anaerobic environment is needed, using an anaerobic jar or anaerobic chamber (glove box) with enriched growth medium, such as non-selective Wilkins Chalgren Agar (WCA) with defibrinated blood (Bird et al. 2002; Bailey & Love 1991). Then isolates can be presumptively identified

using simple phenotypic characterisation techniques, such as colonial morphology, pigmentation, aerobic growth, Gram stain reaction, fluorescence under long-wave UV light (360 nm), production of catalase, enzymatic activity with fluorogenic substrates, and haemagglutination of sheep red cells. These techniques allow the identification of isolates to the genus level or in some cases to the species level (Syed et al. 1980, 1981; Slots & Reynolds 1982; Mallonee et al. 1988; Bird et al. 2002). However, many can only be grouped at the family or genus level. The reliability of culture dependent methods for bacterial identification is not only limited by the fastidious bacterial growth requirements, but also sampling and transport requirements as the bacteria are very sensitive to oxygen exposure (Mackintosh & Colles 1987; Bailey & Love 1991).

In order to identify isolates where phenotypic characteristics are restricted, more advanced molecular methods (such as 16S DNA sequencing of extracted RNA) are required to more accurately identify bacterial isolates. Molecular methods achieve significant specificity through comparison of sequence data to with those recorded in an online database, such as GENBANK (<http://www.ncbi.nlm.nih.gov/genbank>) (Clarridge 2004) or Greengenes (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>) (DeSantis et al. 2006).

The recent introduction of next generation metagenomic sequencing for oral bacterial community profiling provides high throughput sequencing of hundreds of thousands of sequences from single samples, which gives information at an exceptional depth and has allowed understanding of the relationship between microbes and clinical variation (Diaz et al. 2012; Human Microbiome Project 2012; Zaura 2012). This new molecular approach has been applied in dogs (Sturgeon et al. 2013) and cats (Sturgeon et al. 2014) to investigate oral bacterial communities and periodontal plaque ecology with minimal time and cost.

The small number of recently published studies on equine oral microbiology have shown the potential for discovery of novel oral bacteria, which could be the keystone bacteria that cause EPD (Hajishengallis et al. 2012). Most studies have been limited to selected paraoral surfaces, such as tonsillar surfaces (Bailey & Love 1991) or dental abscesses (Mackintosh & Colles 1987), and have not investigated the periodontium. The variety of sites and environmental conditions in the oral cavity of the horse makes the oral microbiota a complex and challenging field of study. By

combining culture dependent phenotypic characterisation methods and molecular methods, such as full chain 16S rRNA sequencing and cloning. A broader range of bacterial diversity within the oral cavity will be able to be identified within a shorter time and at a lower cost.

### **Clinicopathology and assessment of equine periodontal disease**

Apart from studies on the normal anatomical, histological and micro anatomy of equine dental structures and periodontium by the German research groups (Staszuk & Gasse 2004a; 2004d; Staszuk et al. 2005; Staszuk & Gasse 2005; Masset et al. 2006a, 2006c; Staszuk et al. 2006; Warhonowicz et al. 2006; Staszuk & Gasse 2007; Warhonowicz et al. 2007; Huthmann et al. 2009), there are limited studies reporting on histopathological and radiographic changes in equine periodontal disease. The recent and only dedicated study on the histopathological aspect of EPD was by Cox et al. (2012). This study demonstrated that gingival hyperplasia increased with increased periodontal disease. In addition, interdental alveolar bone remodelling was commonly, but not significantly, associated with periodontal disease.

The basic assessment of periodontal status in animals such dogs and cats was adapted from human studies. These assessments involve measuring the periodontal pocket depth and examining the animal for furcation exposures where severe alveolar bone loss is present. Horse cheek teeth, being primarily hypsodont, are not directly comparable to brachydont animals because the tooth root length in hypsodont teeth varies by age. Klugh (2005a) described a method for determining the stage of periodontal disease in horses, which was adapted from a small animal practice, and used percentage of attachment lost and an index of mobility. However, probing depth on its own cannot be used to estimate the extent of PD as the length of the reserve crown and tooth roots in a particular horse is not knowable from visual and/or palpation examination. The use of more advance diagnostic techniques, such as conventional radiographic imaging, Computed tomography (CT), and Magnetic resonance imaging (MRI) are essential.

## **Equine dental radiography**

Equine head and dental anatomy is complex. Some areas, such as the apices, reserve crowns and periodontal structures are impossible to examine in the horse even with the aid of sophisticated instrumentations, such as full mouth speculums, dental mirrors, extreme bright dental lights, retractors and endoscopes. Radiography is considered to be an accurate alternative diagnostic technique for assessment of these hard to examine areas of equine head, especially using advanced imaging techniques, such as CT, standing CT (Porter & Werpy 2014), MRI (Tucker & Farrell 2001), nuclear scintigraphy (Weller 2001; Archer 2003) and computerized 3D reconstructed imaging (Brinkschulte et al. 2014). However, the expense and risks associated with general anaesthesia necessary for CT and MRI will render radiography as the most commonly used ancillary imaging diagnostic technique for equine dental disorders.

### **Computed tomography of horse teeth**

CT is an imaging technology that produces virtual slices or tomographic images from radiography files. CT allows users to view the internal structure of a scanned object. CT in equine practice has become more common in the past decade, due to new generation CT with shorter scan times and improved detail. The advantages of CT over standard radiography is the visualisation of the image region of interest without distortion or overlapping of adjacent structures. Increased sensitivity of CT to visualise different tissues of various radiodensity allows for the measurement of the interdental alveolar process space, the PDL space and length (Barbee et al. 1987; Park et al. 2013). Other advantages of CT over standard radiography is the ability to create virtual 3D reconstructed images by stacking the image slices and adding different colours to various threshold components which can highlight structures such as crestal bone, PDL and teeth. Brinkschulte et al. (2014) used 3D reconstructed imagery to study horse sinonasal communication, which plays a major role in the development of sinusitis. This study achieved excellent spatial definition of these complex anatomical structures, which is an important prerequisite for diagnostic and surgical interventions. In addition Schrock et al. (2014) studied cementum of the equine incisor using  $\mu$ CT to show that incisors are able to compensate for occlusal wear for a limited period of time and may relate to the incident of periodontal

destructive disease such equine odontoclastic tooth resorptive lesion and hypercementosis.

### **Equine periodontal radiography technique**

In human dentistry, radiographs have been widely accepted as auxiliary tools to establish diagnosis and treatment guidelines for patients with PD (Tugnait et al. 2000, 2004; Corbet et al. 2009). As well, in small animal veterinary dental practice, where general anaesthesia is required as part of routine dental examination and treatment, radiographs are highly recommended in all cases presenting with periodontal disease for the success of therapy (Verstraete et al. 1998a; Verstraete et al. 1998b; Farcas et al. 2014). In equine practice there are three common radiography systems: conventional wet film preparation (WF), Computed radiography (CR) and direct digital radiography (DR). The difference between CR and DR is that CR is still film based. In CR, a photostimulable phosphor image plate is used to capture X-ray energy and scanned with a helium neon laser and the emitted light is then converted to digital image photo. DR captures X-ray energy using a semiconductor base sensor and transfers X-ray energy directly into digital signals. By negating the scanning process DR turn-over time is much shorter than CR (Bansal 2006). In small animal dental radiography, CR and DR have been widely used intraorally. The use of equine intraoral radiography with normal size cassette are more practical for incisor and canine as they can be easier for cassette placement and adjust the beam angle. This intraoral radiography technique found to be essential for evaluation of incisor and canine disorder such EOTRH (Earley & Rawlinson 2013). However, equine cheek teeth radiography is mainly limited to extraoral techniques due to equipment availability, patient size and cooperation, and the need for sedation.

The equine extraoral radiography technique has been described by Barakzai and Dixon (2003) where they employed a standing extraoral open mouth (EO) radiographic projection by inserting a PVC pipe between the incisors. This technique produced improved radiographic views of the crown and provided a more accurate diagnosis of dental lesions. Nevertheless, superimposition of the opposite arcade and distortion of the interested tooth often interfere with clear visualisation. In human and small animal dental practice, intraoral radiography (IO) has overcome this limitation by using the bisecting angle technique. In equine dental radiography practice, IO has been

attempted for over a decade but restricted to wet film or a customised computed radiography (CR) plate and the need for strong sedation or even general anaesthesia, which makes this technique less convenient for routine use (O'Brien 1996; Klugh 2005f). By combining advanced CR, modern sedation, and use of a crush IO is now possible for everyday use. A group of Swedish veterinarians designed a custom modified CR plate for use with the bisectonal angle technique for IO in horses and achieved good image results, except for the last molar of the upper cheek teeth arcade (Rubin et al. 2008). However, there were some difficulties with the image plate being damaged by tooth margins.

### **Radiographic evaluation of equine periodontium**

A clear understanding of dental anatomy is essential for radiographic interpretation of EPD. Clinically, EPD begins with inflammation of gingiva (gingivitis). If untreated the disease will progress apically to periodontitis that involves the supporting structures, such as the PDL, alveolar bone, and cancellous bone. The radiographic signs of periodontal disease include discontinuation of lamina dura (present as a thin radiopaque border next to the PDL and alveolar crest), wedge shaped widening of the PDL, reduced crestal bone height, inter radicular bone loss (furcation lesion), and the presence of DI (Tsugawa & Verstraete 2000; Barakzai & Dixon 2003). As EPD is an episodic and cyclical disease, radiographic images showing bone loss may not represent the current stage of the disease.

Radiography can be helpful in diagnosing EPD, determining the prognosis of the disease, and evaluating the outcome of treatment. But radiography is an adjunct to the clinical examination, not a substitution, as it only shows the effect of past bone and tooth remodelling not the current activity.

### **Chemotherapeutic mouth rinse as control and prevention of equine periodontal disease**

Accumulation of plaque initiates gingivitis which may progress to periodontitis if untreated. Periodontal disease is not a true infectious disease but a chronic inflammatory disease due to the periodontopathogens that are part of the normal microbiota. The reversibility of oral plaque ecology mediated by the response to host and environment factors (Marsh 2003) has demonstrated that by maintaining a good

oral health in this ecosystem periodontal disease can be prevented or cured. There are two main methods of periodontal plaque control: mechanical control that reduces the accumulation of plaque, such as by teeth brushing, scaling and root planing, or the use of chemical control, such as an antiseptic oral rinse or mouth wash. In small animal veterinary practice, the brushing of animal teeth is always recommended while scaling, considered more invasive, requires the patient to be under general anaesthesia. By contrast, the recommendation for working equine patients is to have regular dental examinations, especially with geriatric horses (Anthony et al. 2010). In severe EPD cases, more invasive treatments are recommended, such as periodontal flushing, mechanical widening of the DI, or extraction of diseased teeth (Collins & Dixon 2005; Dixon et al. 2008). However, these techniques require special tools, moderate to heavy sedation and should be performed only by experienced veterinary practitioners. In order to promote preventive dentistry, EPD control methods must be easily implemented by everyone with minimal rejection by the patient. Chemical control using an oral rinse or mouth wash has the potential to be effective, as it can be applied by anyone with some degree of horsemanship.

### **Antimicrobial plaque control**

The use of oral chemotherapy was introduced many years ago (Loesche 1976; Johnson & Rozanis 1979). The main purpose of a chemotherapeutic agent is to control the oral plaque or periodontopathogenic bacteria and return a symbiotic state (Hajishengallis & Lamont 2012; Marsh 1994; Moore & Moore 1994). Only a few topical chemotherapy agents, registered internationally for periodontal disease, are used in animals.

#### **Doxycycline hydrochloride (DHL)**

An *in-vitro* study demonstrated that DHL has long acting antibacterial activity against anaerobic bacteria (Gram positive and Gram negative), including polymicrobial oral infections (Terranova et al. 1986; Demirel et al. 1991). DHL is available as a veterinary medical product formulated as a flowable polymer that rapidly hardens when exposed to water and remains in a periodontal pocket for several weeks (Doxirobe® Gel, Zoetis, New Jersey, U.S.A.). In DHL treated dogs with periodontitis,



a substantial improvement in periodontal attachment, reduced periodontal pocket depth and bleeding was shown after 6 weeks to 4 months (Polson et al. 1996; Zetner & Rothmueller 2002).

#### Clindamycin (CLM)

An *in-vitro* study of CLM showed broad spectrum activity against many periodontal pathogens and had good tissue and bone penetration (Baird et al. 1978; Mueller et al. 1999). The clinical trial of CLM in canine periodontal disease post–prophylaxis demonstrated that CLM can reduce the symptoms of PD with less gingival bleeding, reduced periodontal pocket depth, improvement of halitosis, and a reduction of dental plaque and calculus formation (Bowersock et al. 2000; Nielsen et al. 2000; Warrick et al. 2000; Johnston et al. 2011).

#### Chlorhexidine gluconate (CHX)

CHX is the most studied bisguanide antiseptic, with most information on toxicology (Addy 1986). CHX was introduced and has been widely used in both human and veterinary medicine since 1953. It has broad spectrum antimicrobial effect, including against aerobes and anaerobes (Davies et al. 1954; Rindom Schiøtt et al. 1970; Sreenivasan & Gittins 2004). CHX also can reduce the adherence of *P. gingivalis* to epithelial cells by inhibiting the haemagglutination process (Grenier 1996, 2013). CHX has low toxicity and is considered very safe for long term use (Foulkes 1973). Nevertheless, a recent study by Giannelli et al. (2008) showed that CHX is highly cytotoxic to cultured cells and recommended that it be used with caution. The most common side effect is an extrinsic stain on the teeth, but this can be removed by routine dental scaling (Addy 1986). There is limited literature on the effect of CHX in animals apart from dogs used as an experimental staining model (Schemehorn et al. 1982).

#### CHX for EPD prevention and control

As discussed above, current preventative therapies for EPD involve either mechanical or chemical methods, and mechanical methods require veterinarians to perform them as they involve sedation of the patient. According to Australian veterinary medicines and pesticides (<http://apvma.gov.au>) there is only one chemotherapeutic agent registered for topical+oral use in dogs, cats and horses, which is CHX. This

commercially available treatment is easy to apply by an owner and is usually well accepted by the patient. However, there is a lack of scientific data on the product's antibacterial activity against periodontal pathogens.

## **Conclusion**

The study of periodontal disease as part of equine dentistry is an overlooked field of study, which needs more research to clearly understand the nature of the disease and to provide for a good quality of life for horses. There have been no oral pathology surveys reported in horses, so the extent of this problem in wild and domestic horses is unknown. Australia is believed to have the world's largest feral horse population, with approximately one million horses throughout the continent. These horses could provide valuable insight into the effects of feeding on dental health, but the access to live animals is limited due to their remote location. Abattoir surveys provide an opportunity for large scale research data collection without the attendant difficulties associated with general anaesthesia or sedation of horses, or ethical issues associated with trapping and examining wild horses.

The aetiology of equine EPD is yet to be clarified; it is currently unknown whether the periodontopathogens in horses are similar or different to those in other species. The use of culture dependent methods alone will not answer this specific question. Combining conventional culture dependent techniques and new molecular techniques, such as NGS should produce more detailed information on equine oral microbiology and may potentially lead to valuable insights into the aetiology of EPD.

From a clinical point of view, the diagnosis and prognosis of EPD treatment must be achieved at the highest level of accuracy possible. Taking physical measurements by probing depth, as used in small animals, may not be applicable to horses because their dentition is different. The introduction of advanced radiographic techniques, such as intraoral radiography using CR and CT with 3D image reconstruction, holds significant promise for diagnosing and treating EPD as they can provide the precise location and extent of the lesion. Nevertheless, there is limited information available on these radiographic techniques and how to interpret intraoral radiographic images of EPD in horses.

The treatment of EPD is challenging as the disease itself is not primarily infectious but chronic inflammation, hence systemic antibiotics may not be the best approach, as long term use may disturb to gut microbiota leading to secondary symptoms such diarrhoea or colic. The use an oral antiseptic agent has been studied in many animals and humans with proven success for the treatment and control of periodontal disease. One of the best studied oral antiseptic agents is CHX, but its efficacy in horses has not been reported.

**Chapter 3:**  
**An abattoir survey of equine dental abnormalities in**  
**South-East Queensland, Australia**

## Introduction

Dental diseases are very common oral disorders in horses (Dixon & Dacre 2005). An early survey reported that 10% of cases presented to equine practices in England were dental related (Cook 1965) and dental cases were in the top five medical problems in adult horses in the U.S. (Traub-Dargatz et al. 1991). In the classic study by Dixon and co-workers of 400 horses referred to an equine clinic in Scotland because of dental disorders, 87% of cases were diagnosed with primary disorders of the cheek teeth, including 44 cases (12.6%) with gross abnormalities of wear of which 5 (11.4%) surprisingly exhibited no clinical signs while other types of disorder such as traumatic damage, idiopathic fractures and dental tumours always presented with clinical signs (Dixon et al. 2000c). In addition, many dental related surveys from various geographical locations on both live horses and post-mortem specimens have shown clinically significant findings on dental abnormalities in populations of horses (Brigham & Duncanson 2000a, 2000d; Gordon 2001; Anthony et al. 2010; Gere & Dixon 2010). In a survey of 556 horse cadavers examined in a Canadian abattoir, 70% of cases had at least one type of dental abnormality on their cheek teeth. The most common dental disorder found was sharp enamel points (SEP) (47.7%) and 36.2% of horses with SEP had buccal abrasions<sup>7</sup>. However, at this time no targeted detailed equine dental survey has been conducted on an Australian horse population. Australia has a large population of horses, with an estimated number of 1.2 million (Gordon 2001). McGowan et al. undertook a general health questionnaire with owners of horses aged 15 years or older in Queensland, Australia (McGowan et al. 2010). They found that 9% of horses had clinical signs of dental disease reported by their owners, which may contribute to weight loss, head and mouth pain, behavioural abnormalities and poor performance.

In Queensland, aged, unwanted or retired horses from a broad range of sources are sent to abattoir for slaughter. The aim of this study was to investigate the prevalence and association of dental disorders in a population of horses presented for slaughter.

## **Materials and methods**

### **Study design and horses**

The study was a cross sectional survey of dental abnormalities in horses, conducted from November 2011 to January 2012 at an abattoir in Caboolture, Queensland, Australia which exports horse meat for human consumption. The catchment area for this establishment is mainly South-East Queensland. Horses are of various breeds including Thoroughbred, Standardbred, Stock horses and mixed/cross breed. Sick or severely injured horses are not accepted for slaughter. The survey was conducted fortnightly. All horses for a particular day were studied. The study was approved by the Animal ethics committee of the University of Queensland (ANRFA/SVS/331/13).

### **Head and dental examination**

The heads were examined at the abattoir premises. The tongue and most of the facial and masticatory muscles, lymph nodes, and salivary glands were removed during the slaughter process. The examination of the heads for abnormalities started with visual examination of skull symmetry followed by flushing of the oral cavity to clear all debris to increase visibility.

Horses were grouped in four age groups (0-5 years, 6-10 years, 11-15 years and over 15 years) using the appearance of dentition. Stage of teeth eruption for horses aged <5 years old; loss of dental cups on incisors and shape of occlusal table for horses aged 6-10 years; persistence of central enamel for horses aged 11-15 years and the loss of central enamel for horses aged 16 years and over. The detailed criteria for aging horses by dentition are described elsewhere (Richardson et al. 1995; Muylle et al. 1996).

Dental examination started with incisor abnormalities such as bite alignment (which was categorised into normal alignment, overshot/parrot mouth and undershot/sow mouth) and presence of canine abnormalities by following the definition described by Dixon et al. (1999a). The cheek teeth were closely examined on all surfaces (buccal, palatal, lingual, labial and occlusal) and checked for the presence of wolf teeth (first premolar). The gingivitis index could not be defined in this study due

to post-mortem gingival discolouration and lack of gingival bleeding on probing. Only visual PP (food and material packed into the gingival crevice) and diastemata (visible spacing between adjacent teeth) were recorded. All dental examinations were made using both visual and palpation examination techniques.

The data recorded consisted of dental age of the horse, presence of canines and wolf teeth (first premolar), presence of SEP (where > 1mm was observed from level of occlusal surface), PP, diastemata, bite alignment, mobile teeth, fractured teeth, abnormalities of wear and eruption such as stepped teeth, hooks, ramps and exaggerated transverse ridges (ETR). The description of dental abnormalities can be found elsewhere (Anthony et al. 2010; Dixon and Dacre 2005; Dixon et al. 1999a; Dixon et al 1999b; Dixon et al. 2000c; Simhofer et al. 2008).



### **Dental abnormalities recording and charting**

All the findings were recorded using voice recorder in the working environment, then transferred to a dental chart using the Modified Triadan tooth numbering system (Floyd 1991) as shown in Figure 2.

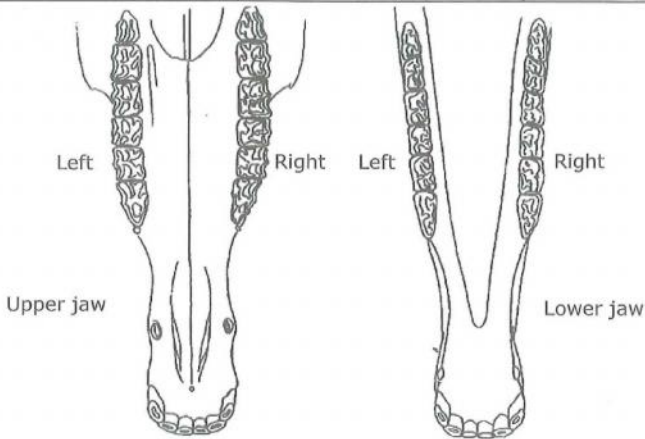
### **Statistics**

The percentage prevalence (number of heads with dental abnormalities divided by the number of heads examined x 100) of dental abnormalities were estimated with a 95% confidence interval. Association with age group was evaluated using Chi square test, or Fisher's exact test if the cell frequency was <5. Strength of association was evaluated using Cramer's V, where >0.5 indicates high association, 0.3-0.5 moderate association, 0.1-0.3 low association and 0-0.1 little if any association.

The average age of affected and non-affected was compared using a t-test for independent samples. SPSS (Version 20) was used for analysis.


 <b>Equine Veterinarians Australia</b> <small>A Special Interest Group of the Australian Veterinary Association Ltd. ABN 63 008 522 852</small>			
Owner/Trainer:		Address:	
Name of horse:		Age:	
Colour:	Breed:	Sex:	
Microchip:	Brands:	L	R
Date:	Last dental:	Condition:	
<b>Riding history:</b> <input type="checkbox"/> Bit used <input type="checkbox"/> Chews bit <input type="checkbox"/> Bit resistance   L   R <input type="checkbox"/> Head tossing <input type="checkbox"/> Other:			
<b>Clinical history:</b> <input type="checkbox"/> Wt loss <input type="checkbox"/> Spills feed <input type="checkbox"/> Colic <input type="checkbox"/> Slow eater <input type="checkbox"/> Quidding <input type="checkbox"/> Undigested feed in faeces <input type="checkbox"/> Washes feed in water			
<b>Examination:</b>	Occlusion:	Lateral Excursion:	Flexion Test:




L. Upper jaw  
☐☐☐☐☐☐



R. Upper jaw  
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L. Lower jaw  
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R. Lower jaw  
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**Comment / Treatment / Plan:**

**Recommended revisit date:**    /    /

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Address:		Place stamp here:	
AVA No:	VPB No:		

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**Figure 2** Equine veterinarians Australia's equine dental chart used as the dental chart for this study.



## **Results**

### **General observation**

Due to the protocol and safety concerns for visitors at the abattoir, the examiner was not allowed access to the holding area to examine the live horses for gender and general health. It was observed from a distance that horses were mixed of various breeds such as Standardbred, Thoroughbred, Stock horses and mixed/cross breed. There were no feral horses in this survey. All of the horses appeared to be healthy as sick or severely injured horses are excluded from the slaughter process.

### **Dental abnormalities**

Four hundred heads were examined and all were included in the analyses. Dental age ranged from 1-30 years (median, 8 years), with 134 (33.5%) horses aged 0-5 years, 97, (24.3%) 6-10 years, 120 (30%) 11-15 years and 49 (12.3%) >15 years. No skull asymmetry was observed. However, masticatory muscular asymmetry could not be evaluated due to removal in the slaughter process. A total of 375 heads (93.8%) were found to have one or more dental abnormalities and 25 heads (6.7%) were without any dental abnormalities. Details of the frequency of dental abnormalities are shown in Table 1.

Frequency of incisors bite alignment, canine abnormalities and presence of wolf teeth

The frequency of incisor alignment, defined as normal bite alignment, was 79.9% of the 400 horse heads with a 95% confidence interval (CI) of 75.5-83.6%; overshoot was seen in 20.0% (95% CI 16.2-24.3%) of the heads and no undershot was observed. Two fractured canines were found at 204 and 304 and 22.75% (95% CI 18.7-27.1 %) of horses had one or more wolf teeth.

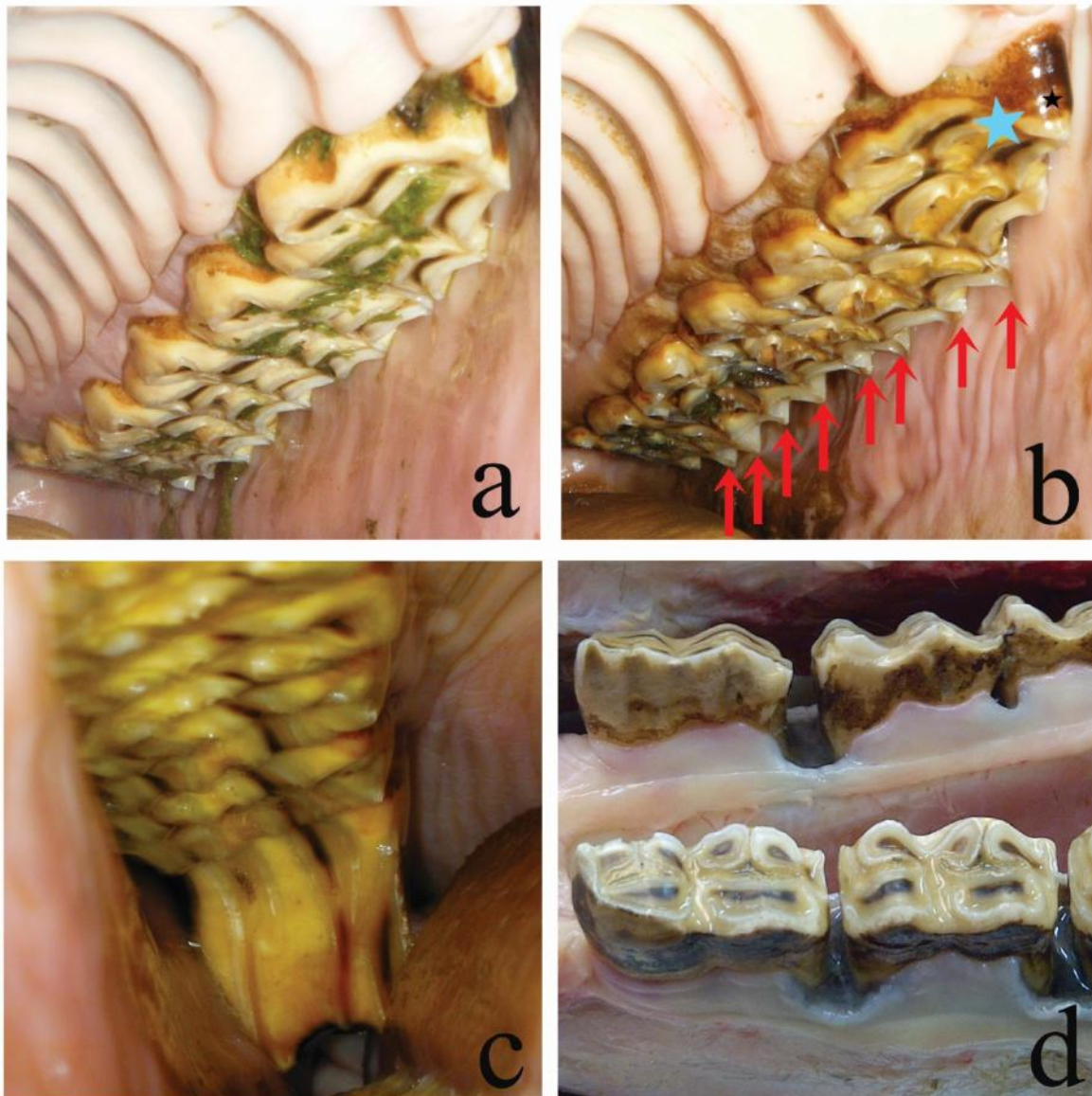
**Table 1** Frequency of dental abnormalities observed in 400 horse skulls from South-East Queensland, Australia

Dental Abnormality	No. of horses	Proportion (%)	95% CI (%)
No abnormality observed	25	6.2	4.1–9.1
SEP	272	68	63.4–72.6
Hook	172	43	38–48
Wave mouth	102	25.5	21.3–30.0
Periodontal pocket	89	22.3	18.3–26.7
ETR	54	13.5	10.3–17.2
Diastema	52	13	9.9–16.7
Ramp	50	12.5	9.4–16.1
Retained deciduous	44	11	8.1–14.5
Stepped tooth	34	8.5	6–11.7
Missing tooth	21	5.3	3.3–7.9
Fractured tooth	16	4	2.3–6.4
Mobile tooth	11	2.8	1.4–4.9
Cupped out tooth	49	12.3	9.0–15.5

CI, confidence interval; ETR, exaggerated transverse ridges; SEP, sharp enamel points

### **Abnormalities of wear and overgrowth**

The normal occlusal surface of a young horse is shown in Figure 2a and abnormalities of wear and overgrowth such as SEP, hook, stepped teeth and periodontal pockets are shown in Figure 2b-d. A total of 98.4% (95% CI 96.5-99.4%) of hooks were found in the cheek teeth; 29.7% (95% CI 15.9-47.0%) of stepped teeth were found at the lower left mandibular fourth premolar (308) and 35.1% (95% CI 20.2-52.5%) were found at the lower right mandibular fourth premolar (408). In addition, 40% (95% CI 29.5-50.2%) of ramps were found in the lower left mandibular second premolars (306) and 38.8% (95% CI 28.4-50.0%) were found at the lower right mandibular second premolar (406). More descriptions on dental abnormalities are described elsewhere<sup>1</sup>.



**Figure 3** (a) Normal teeth occlusion in 5 year old horse, (b) sharp enamel point (arrows) and hook (star) in 6 year old horse, (c) stepped tooth in 12 year old horse and (d) diastemata/periodontal pockets with visible soft tissue and bone loss in miniature pony.

### **Periodontal pockets and diastema**

There were 144 periodontal pockets observed in 89 heads found mostly interproximally (between two adjacent teeth) and only one periodontal pocket was found on the palatal side of the upper left fourth premolar (208). Prevalence of periodontal pockets in the incisor area was 2.8% (95% CI 0.8-6.9%), the premolar area was 22.2% (95% CI 15.7-30.0%), the interdental space between the fourth premolar and the first molar was 41.67% (95% CI 33.5-50.2%) and the molar area was 32.64% (95% CI 25.1-40.9 %), the premolar area 22.2% (95% CI 15.7-30%) and the incisor area 2.8% (95% CI 0.8-6.9%) (Figure 4).

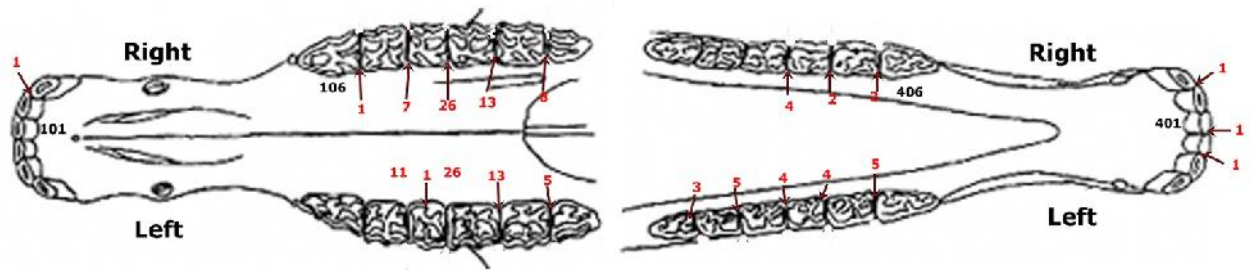
A total of 107 diastemata were found in 52 heads, 40.2% (95% CI 30.8-50.1%) were interproximally located between incisors (Figure 5). Thirteen mobile teeth were found with no specific location in the mouth.

### **Age and dental abnormalities**

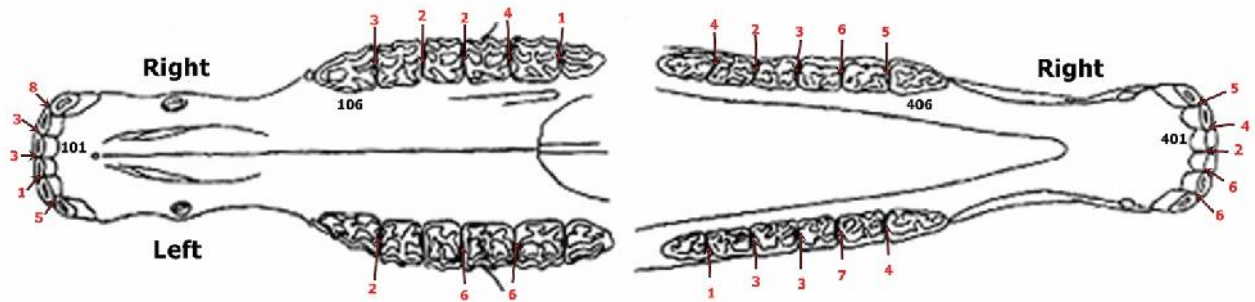
As expected the proportion of abnormalities differed according to the age group. The 0-5 year age group had a higher prevalence of retained deciduous teeth and ETR (Figure 5a) than any other age group (Table 1).

The 11-15 years age group had a prevalence of hooks of 59.2% (95% CI 49.8-68.1%) with an odds ratio (OR) 3.7 times higher than reference group (0-5 years horses) (Table 2); 30.8% (95% CI 22.7-39.9%) had wave mouth (Figure 6b); 12.5% (95% CI 7.2-19.8%) had stepped tooth; 14.2% (8.5-21.7%) had ramps and 6.7% (2.9-12.7%) had fractured teeth (Figure 6c).

The over 15 year group of horses had a prevalence of diastemata of 40.8% (27.0-55.8%) with an OR of 7.7 much higher than reference group; 12.2% (4.6-24.8%) had mobile tooth and 18.4% (8.8-32.2%) had missing teeth with a OR of 7.3 much time higher than reference group (Table 2).



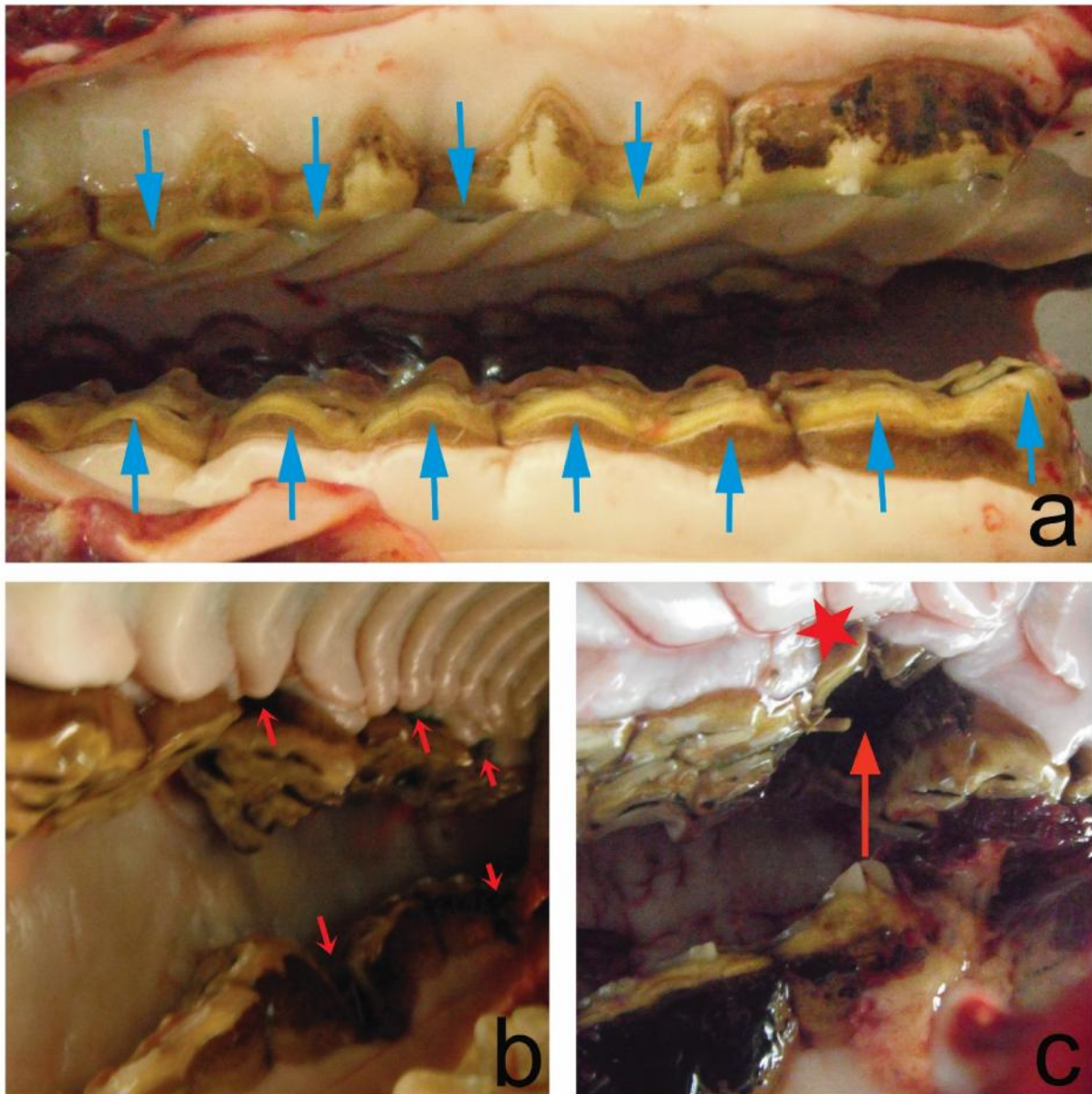
**Figure 4** The result of periodontal pockets distribution between horses teeth from 89 heads represents the number of 144 periodontal pockets found at each location.



**Figure 5** The result of diastema distribution between horses teeth from 52 heads represent the number of diastemata found at each location with total of 107 diastemata.



Comparison of proportions by chi-square test for each group showed that age group had no effect on the presence of SEP, ramp teeth, fractured teeth and peripheral caries but that age group was related to all other abnormalities (Table 2). Not all abnormalities increased linearly with age, where wave mouth and diastema were less common in the 5-10 year old horses. According to the results of Cramer's V test a high association was shown only with diastema and cupped out (Table 2).



**Figure 6** Equine dental abnormalities (a) Exaggerated transverse ridges (blue arrow) in a 5 years old horse, (b) wave mouth with severe periodontal disease (red arrows), (c) Fracture in 109 with oronasal fistula (star and red arrows indicates the remnants of fractured tooth, with oronasal fistula, respectively).

## Discussion

The results of this study showed that 93.8 % of horses examined had at least one dental abnormality. The most common abnormality found was SEP (68%).

There were major variations in prevalence of SEP from previous reported studies ranging from half of the population to near 100% of the population (Brigham & Duncanson 2000a, 2000d; Simhofer et al. 2008; Anthony et al. 2010). This may be due to different definitions of the SEP and examination criteria in each study. However, due to the high prevalence of SEP reported in many studies (Simhofer et al. 2008; Anthony et al. 2010; O'Neill et al. 2010; Pathomsakulwon et al. 2011), it could be considered as a normal physiological change caused by routine eating and grinding behaviour or individual anatomic variation of large vertical ridges (cingule). Unless these SEP are causing lacerations or other types of wounds to the surrounding soft tissue, for instance, buccal mucosa and tongue, then it may not be considered as an abnormality. In our study 32% of studied horses showed no SEP which could not be justified. It is not known whether this was due to human intervention, or whether it is due to natural wear and having a dental history would determine which one. The variations in the results may be accounted for by differences in methods and tools used for floating horse teeth used by veterinarians or lay persons. The overgrowths of teeth were clearly site specific, for example hooks and ramps are most likely to be found on the rostral or caudal tooth of the cheek teeth arcades rather than other teeth. The stepped teeth were commonly present at the mandibular fourth premolar; this may be due to the order in the eruption of cheek teeth where the last tooth to erupt is the fourth premolar which may involve other pathologies such as a retained deciduous fourth premolar and entrapment of the permanent tooth. Further investigation is required on cause and relationship of the overgrowth type of abnormality to other malocclusions such as overshot and undershot bite alignment.

The result of incisor alignment shows that there was a higher prevalence of overshot (parrot mouth) than in the Canadian abattoir survey (0.4%) (Anthony et al. 2010). This might reflect differences in the definition of the malocclusion, genetic or environmental factors as the studies were conducted in different geographical locations. There were some abnormalities such as mobile and fractured teeth which were found without specific location possibly because of low prevalence.



The visible periodontal pockets were more likely to be found further caudal in the oral cavity such as between the fourth premolar (108, 208, 308, 408) and the first molar (109, 209, 309, 409) or other molars. This more caudal area has less movement of the tongue to shift food particles to the teeth for mastication. This lack of tongue movement may increase the chance of food particles becoming trapped interproximally. The correlation between malocclusion as a risk factor for periodontal disease (PD) and occurrence of PD in each age group is a holy grail. However, the modelling of this data is complex where one type of malocclusion or a multifactorial pathology may be linked to occurrence of PD. In addition, age variation is also another factor to be considered. Diastemata were commonly found between incisors and cheek teeth of geriatric horses and this may be due to the wedge shape of geriatric teeth that creates spaces between two adjacent teeth. Moreover, the result of Cramer's V test has shown that diastemata have a moderate association with age group. The incidence of diastemata was significantly higher in 0-5 years old and >15 year old groups (8 times higher than the reference group). The increased prevalence in young horses may be physiological, due to ongoing tooth eruption processes that create space which allow feed particles to be trapped. However, this is different for the old horse where tooth eruption has already finished and hence the diastema in an old horse is an acquired disease.

The horse teeth have tight compression at the occlusal surface. However, as the horse gets older this tight space widens with the wear of occlusal surfaces especially in cheek teeth, which creates spaces due to tapering of the tooth called valve diastema as described in previous studies (Carmalt 2003; Dixon & Dacre 2005). These spaces are common areas for accumulation of feed such as grass and hay, which provides for a suitable environment to allow the indigenous anaerobic Gram-negative oral bacteria to grow. Subsequently this will initiate the process as seen in periodontitis, which is involved in loss of supporting structure such as periodontal ligament, the gum and alveolar bone (Dixon & Dacre 2005; Staszuk et al. 2008; Cox et al. 2012). However it has been suggested that this periodontitis could be reversible due to the continuous eruption of equine teeth (Cox et al. 2012).

The results from our study showed that the 10 to 15 year old group had at least one type of dental abnormality, which has also been reported in the Canadian abattoir study. However, the authors of this paper did not detail the incidences of the various types of dental abnormalities versus age (Anthony et al. 2010). The abnormalities that

involve eruption of the teeth such as ETR and retained deciduous tooth are more likely to be found in the 0-5 year age group, which may be due to the active eruption process of the teeth. The high prevalence of retained deciduous in 0-5 year old group may be considered a normal finding due to rate and time of eruption of the permanent tooth. However, a long term investigation on ETR needs to be done before accepting ETR as a normal wear pattern for this age group. ETR may lead to more severe dental diseases such as diastema and PD, due to ETR forcing feed interproximally in the opposing arcade during mastication. One incident of peripheral caries was found between 109 and 110 in a 10 year old horse. This may be due to differences in nutrients in local feed, feeding practices or soil type in the Queensland region (Gere and Dixon 2010). In addition, cupped out teeth has the highest association among observed abnormalities. This could due to normal physiological changes of teeth structure where the infundibulae have lost their enamel layer and left only soft dentine which will wear quicker resulting in the oyster shape or cupped out. This physiological change may need to be considered normal in the senior horse with age more than 16 years old.

Preventive dentistry introduced from a young age could correct the dental problems often missed by the horse owner and therefore prevent these problems becoming clinically significant. In addition, there appears to be a lack of appreciation by horse owners of the signs of dental abnormalities (McGowan et al. 2010). Many studies have recommended annually dental examinations for horses aged over 15 years, but our study show that horses in all age groups had abnormalities that can be easily identified during a complete routine dental examination with minimal equipment. The interval of dental examination and treatment will vary with individual horses due to different work discipline, diet, congenital or acquired abnormalities, practice experience and equipment used. In order to prevent more severe abnormalities, a routine dental examination should be given to domesticated horses in all age groups.

The results of this study will provide some predictions on what abnormalities are likely to be found in each age group. Further studies are required into the relationships between each dental abnormality and its clinical consequences.

A study of feral horse populations is likely to provide significant data on dental abnormalities. These horses are on natural diet and lack of human intervention and

will exhibit dental abnormalities associated with normal masticatory wear. No feral horses were included in this study. Thus access to feral horses for dental research would be highly valuable for future equine dental research.

### **Limitations of the study**

Dental aging has limits of accuracy, but true age was difficult to determine because of the abattoir's work protocols. The method of age determination in horses by their dentition has been used for >100 years with acceptable accuracy, in horses aged <5 years due to the process of eruption of teeth (Richardson et al. 1995). Age estimation is less accurate as the age increases due to reliance on occlusal wear patterns. Studies of equine dental age were based on changes of teeth structures in large populations of normal horses. There are some populations where the teeth do not undergo the normal changes of dentition; these factors make the dental age less accurate, but still relatively acceptable. Age grouping by estimated dental age remains the best approach for statistical analysis of age related dental abnormalities.

The second limitation was the recognition of periodontal pocket depth by visual gross anatomic change. The significance of pocket depth measurement was difficult to interpret due to inability to determine total loss of attachment without using radiographic images of the teeth (Gieche 2010). Radiation health risk precluded use of this modality in the abattoir work environment.

The third limitation was the removal of facial muscles, cheek and tongue. This removal limited the assessment of facial muscle symmetry, ulceration of soft tissue which may show the clearer correlation to some of abnormalities such as SEP and fractured tooth.

**Table 2** Prevalence of dental abnormalities in percentage of age groups with Pearson chi-square and Cramer's V test.

	<b>0–5 yr (N = 134)</b>	<b>6–10 yr (N = 97)</b>	<b>11–15 yr (N = 120)</b>	<b>&gt;15 yr (N = 49)</b>	<b>Chi square</b>	<b>P value</b>	<b>Cramer's V (Sig)</b>
	n (%) <sup>a</sup>	n (%) <sup>a</sup>	n (%) <sup>a</sup>	n (%) <sup>a</sup>			
SEP	84 (30.9)	73 (26.8)	83 (30.5)	32 (11.8)	4.325	0.228	0.104 (0.228)
Hook	38 (22.1)	44 (25.6)	71 (41.3)	19 (11.0)	25.094	0.000	0.250 (0.000)
Wave mouth	25 (24.5)	15 (14.7)	37 (36.6)	25 (24.5)	27.041	0.000	0.260 (0.000)
Periodontal pocket	21 (23.6)	16 (18.0)	32 (36.0)	20 (22.5)	16.326	0.001	0.202 (0.001)
ETR	27 (50.0)	19 (35.2)	7 (13.0)	1 (1.9)	19.702	0.000	0.222 (0.000)
Diastema	11 (21.2)	3 (5.8)	18 (34.6)	20 (38.5)	45.084	0.000	0.336 (0.000)
Ramp	10 (20.0)	15 (30.0)	17 (34.0)	8 (16.0)	4.89	0.183	0.110 (0.183)
Retrained deciduous	39 (88.6)	3 (6.8)	2 (4.5)	0 (0)	67.792	0.000	0.412 (0.000)
Stepped tooth	4 (11.8)	11 (32.4)	15 (44.1)	4 (11.8)	8.722	0.000	0.148 (0.033)
Missing tooth	4 (19.0)	4 (19.0)	4 (19.0)	9 (42.9)	19.465	0.000	0.221 (0.000)

Fractured tooth	4 (25.0)	2 (12.5)	8 (50.0)	2 (12.5)	3.505	0.320	0.094 (0.320)
Mobile tooth	0 (0)	1 (9.1)	4 (36.4)	6 (54.5)	21.532	0.000	0.232 (0.000)
Cupped out	5 (10.2)	4 (8.2)	9 (18.4)	31 (63.3)	136.159	0.000	0.583 (0.000)
Peripheral caries	0 (0)	1 (100)	0 (0)	0 (0)	3.132	0.372	0.088 (0.372)
One or more abnormalities observed <sup>b</sup>	120 (89.6)	91 (93.8)	117 (97.5)	47 (95.9)	7.304	0.063	0.135 (0.063)

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<sup>a</sup>percentage of all studied groups i.e. the number of horses with hook at the age of 0–5 years was 38 which was 22.1% of all studied horses with hook.

<sup>b</sup>percentage of each estimated age group i.e. the number of horses with at least one or more dental abnormalities at the age of 0–5 years was 120 horses which was 89.6% of observed horses aged 0–5 years of age group.

**Table 3** Association between age and dental abnormality compared to horses age 0-5 years.

Age group (year)	Hook		Wave		Stepped tooth		Periodontal pocket		Diastema		Missing tooth	
	OR <sup>a</sup> (95%CI) <sup>b</sup>	P-value	OR <sup>a</sup> (95%CI)	P-value	OR <sup>a</sup> (95%CI)	P-value	OR <sup>a</sup> (95%CI)	P-value	OR <sup>a</sup> (95%CI)	P-value	OR <sup>a</sup> (95%CI)	P-value
<b>0-5</b>	Reference group											
<b>6-10</b>	2.1 (1.21-3.63)	0.008	0.5 (0.40-1.61)	0.798	0.0 (1.25-13.48)	4.157	1.1 (0.52-2.16)	.866	0.4 (0.97-1.32)	.122	1.4 (0.34-5.73)	.642
<b>11-15</b>	3.7 (2.14-6.18)	0.000	0.0 (1.09-3.48)	1.944	0.008 (1.49-14.40)	4.643	2.0 (1.05-3.62)	.033	2.0 (0.89-4.37)	.094	1.1 (0.27-4.58)	.874
<b>Over 15</b>	1.6 (0.81-3.18)	0.180	0.0 (2.23-9.23)	4.542	0.1 (0.694-12.03)	2.889	3.7 (1.78-7.75)	.000	7.7 (3.33-17.85)	.000	7.3 (2.13-25.02)	.002

<sup>a</sup>OR, odds ratio - for example the odds of having a hook in horses age 6–10 years were 2.1 times than in horses age 0-5 years old); for odds ratio.

<sup>b</sup>95% CI- the 95% confidence interval calculate for each odd ratio.

**Chapter 4:**  
**The microbiome of equine periodontal disease from  
culture dependent and molecular perspective.**

## Introduction

The indigenous microbiota found in the oral cavity of horses, acting as opportunistic pathogens, are the aetiological agents of the oral disease, equine periodontal disease (EPD) (Bailey et al. 1991; Dorsch et al. 2001; Takada et al. 2010). Early colonising Gram-positive bacteria adhere to the enamel surfaces of teeth and late colonising bacteria attach through receptor binding to form a supra-gingival biofilm (Kolenbrander, 2010). This microbial biofilm grows along the gingival sulcus and there are changes in the microbiome towards to a more motile, anaerobic Gram negative bacteria. Bacteria initiate an inflammatory host response in the periodontium with loss of gingival integrity, development and colonisation of a sub-gingival periodontal pocket (Marsh 1994).

Early studies of oral microbiology were based on culture dependent methods. These early methods were limited as not all bacteria in the oral cavity could be cultivated and they failed to demonstrate the diversity of the microbial community (Syed et al. 1980; Syed et al. 1981; Mallonee et al 1988; Baker 1979). Early methods used non-selective media (such as Tryptic Soy agar, WCA, Brucella agar) for cultivation of bacteria under strict anaerobic or microaerophilic conditions (Mackintosh & Colles 1987; Mallonee et al. 1988; Bird et al. 2002).

The oral anaerobic organisms are very sensitive to oxygen. Therefore, samples were transported in reduced transport media (Syed et al. 1980; Mallonee et al. 1988), frozen using dry ice or liquid nitrogen and then cultivated immediately or stored frozen until required (Bird et al. 2002). Bacteria have been cultivated using the Hungate roll-tube technique (Attebery & Finegold 1969), or using anaerobic jars (Bird et al. 2002). With improvements in technology, anaerobic chambers facilitate the handling and culturing of samples in an atmosphere free of oxygen. While some bacterial species are relatively easy to grow and identify, others are more difficult due to limitations in the growth requirements of those bacteria.

The identification of bacteria grown in culture follows many phenotypic methods. With the introduction of molecular methods, bacteria can be identified using 16S rRNA gene sequencing and cloning methods. Molecular methods have played a major role in the identification of bacterial isolates from both human and veterinary clinical



specimens (Clarridge 2004; Spratt 2004; Kato et al. 2011; Riggio et al. 2011). Further technological advances, including next generation sequencing (NGS), have provided even more detailed information about the oral microbiome in humans (Keijser et al. 2008) and animals, such dogs (Sturgeon et al. 2013) and cats (Sturgeon et al. 2014).

The current knowledge of equine oral microbiota has been limited to culture dependent methods, with reported bacterial isolates such as *Peptostreptococcus* spp., *Eubacterium* spp., *Veillonella* spp., *Porphyromonas* spp., *Prevotella* spp., and *Fusobacterium* spp. (Bailey & Love 1991; Dorsch et al. 2001). The aims of this study was to isolate and identify bacteria from equine plaque and then to compare the bacterial diversity between the healthy and periodontitis samples from horses using both culture and molecular methods (high-throughput NGS of the 16S rRNA gene).

## **Materials and methods**

### **Animals**

Horses from the School of Veterinary Science University of Queensland's teaching herd that had no history of dental treatment during the six months prior to the study were recruited. This study was given ethical approval for the use of these horses (AEC approval No. SVS/161/12).

The animals were examined and classified into two groups:

- healthy group (four horses) where no visible or mild gingival inflammation and no periodontal pockets (PP) or of <5 mm pockets were present.
- periodontitis group (four horses) where PP at 6 mm depth were present with visible gingival recession and bone loss and/or bleed upon probing.

### **Collection of dental plaque samples**

Before examination, each horse's mouth was rinsed with water and the sample sites were cleaned with a gentle stream of distilled water. Dental plaque was then collected using No: 90 sterilized paper points, held by 47.5 cm sterilized equine periodontal forceps, placed and held in the sample site for 30 seconds. In the healthy

group, sites selected were the gingival sulcus and in the diseased group, sites were PP or DI. The paper points with plaque sample were immediately transferred into cryovials containing 1 ml of reduced transport medium (WCB with 10% glycerol – see below), and frozen in liquid nitrogen for transport to the laboratory. Samples were stored frozen at -80°C or liquid nitrogen until required for microbial analysis.

Reduced transport medium was prepared using Wilkins Chalgren Broth (WCB) (Oxoid Australia Ltd. West Hedelberg, Victoria, Australia) with glycerol (10%). The medium was autoclaved, cooled to room temperature and 1 mL aliquots transferred into cryovials. The vials were placed in an anaerobic atmosphere of 86% N<sub>2</sub>, 10% CO<sub>2</sub>, 4% H<sub>2</sub> and left overnight to reduce the medium.

### **Bacterial isolation and characterization**

All work was carried out in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, U.S.A.). Frozen samples were thawed quickly and mixed by vortexing for 30 s. Ten-fold serial dilutions ( $10^{-1}$  –  $10^{-6}$ ) of the plaque samples were made in reduced WCB. One hundred microlitres of each dilution was spread onto non-selective WCA) supplemented with 5% defibrinated horse blood and incubated at 37°C in an anaerobic chamber in an atmosphere of 86% N<sub>2</sub>, 10% CO<sub>2</sub>, 4% H<sub>2</sub>. Plates were examined after 4-5 days and re-examined after a further 10 days.

### **Identification of bacterial isolates**

The colonies were examined under a plate microscope and eight to ten morphologically distinct colonies were subcultured to obtain pure cultures for identification and phenotypic characterisation, including growth under aerobic, microaerophilic (5% CO<sub>2</sub>), or anaerobic conditions, colonial morphology, cellular morphology by Gram staining, catalase production, oxidase and motility. All black/brown pigmented colonies were subcultured with a streak of *S. aureus* as a co-culture to enhance the growth and pigmentation. Plates were incubated anaerobically after for 3-4 days (before pigmentation) and the colonies re-examined using a Wood's light (long wavelength UV light; 360 nm). Colonies that appeared red to orange were classified as fluorescence positive (Slots & Reynolds 1982). Following presumptive phenotypic identification bacterial isolates were frozen in WCB containing 10%

glycerol and stored at -80°C. Phenotypic identification of isolates was confirmed by 16S rRNA gene sequencing.

### **DNA extraction and polymerase chain reaction (PCR)**

Bacterial cells from a well isolated colony were selected and harvested for DNA extraction and polymerase chain reaction (PCR). Bacterial DNA was extracted using the manufacturer's protocol (PrepMan® Ultra rRNA Sample Preparation Protocol; PN 4367554) (Applied Biosystems by Life Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia). 16S rRNA genes were amplified with universal primers, **27F (5'-AGAGTTTGATCMTGGCTCAG)** and **1492R (5'-TACGGYTACCCTGTTACGACTT)**. The PCR was performed under the following conditions: 5 minutes initial denaturation at 94°C; 30 cycles of denaturation (30 s at 94°), annealing (30s at 46°C), and extension (2 min at 72°C); a final extension at 72°C for 7 min. The PCR products were kept at 4°C until sequenced at the Animal Genetics Laboratory, School of Veterinary Science University of Queensland, Gatton.

Each 16S PCR product was partially sequenced with the universal sequencing primer **27F (5'-AGAGTTTGATCMTGGCTCAG)** and **1492R (5'-TACGGYTACCCTGTTACGACTT)** and BigDye® Terminators version 3.1 (Applied Biosystems by Life Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia). Sequencing was performed using an automated DNA sequencer (HITACHI 3130xl Genetic Analyzer, Applied Biosystems by Life Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia) according to manufacturer's instructions. Sequences were compiled using the software package GeneStudio Version 2.2 and compared with bacterial 16S rRNA gene sequences from GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### **Next generation metagenomic sequencing**

Bacterial DNA was extracted from each plaque sample using the Powesoil® DNA Isolation Kit (Mobio Laboratory, Carlsbad, CA), following the manufacturer's instructions. The extracted DNA was verified and quantified by Qubit® Fluorometer using the dsDNA BR Assay kit (Life Technology) All DNA extractions, NextGen sequencing and subsequent data processing were performed at the Australian Centre

for Ecogenomics, School of Chemistry and Molecular Bioscience, The University of Queensland, St Lucia.

The 16S rRNA gene encompassing the V6 to V8 regions was targeted using the **803F (5'-TTAGAKACCCBNGTAGTC-3')** and **1392wR (5'-ACGGGCGGTGWGTRC-3')** primers (Kunin et al. 2010) modified at the end to contain an Illumina specific adapter sequence (**803F:5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGAKACCCBNGTAGTC3'** and **1392wR: 5'GTCTCGTGGGCTCGGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGGGCGGTGWGTRC3'**).

16S rRNA library preparation workflow from Illumina (#15044223 Rev.B) was performed as follows. Initially, PCR products of ~590bp were amplified according to the specified workflow but using Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs) in standard PCR reactions. DNA purification of PCR products at each stage was performed using Agencourt AMPure XP beads (Beckman Coulter). Purified DNA was indexed with unique 8bp barcodes using the Illumina Nextera XT v2 Index Kit A-D (Illumina FC-131-1002) in standard PCR conditions with Q5 Hot Start High-Fidelity 2X Master Mix. Indexed amplicons were pooled together in equimolar concentrations and sequenced on MiSeq Sequencing System (Illumina) using paired end sequencing with V3 300bp chemistry according to manufacturer's protocol.

### **Next generation sequencing data processing**

Quality Control was performed using the Illumina MiSeq platform. Fastq files were processed with Fastqc (Andrews 2010). Primer sequences were removed, and poor quality sequences were trimmed using Trimmomatic (Bolger et al. 2014). All reads were trimmed to 250 bases, reads with less than 250 bp were removed. Operational taxonomic units (OTUs) were selected using QIIME (Caporaso et al. 2010) with a 97% similarity threshold. OTUs with less than 0.05% relative abundance in the OTUs table were removed. Representative OTU sequences were annotated by BLASTing against the Greengenes reference database (version 2013/05, [http://greengenes.lbl.gov/cgi-bin/nph-blast\\_interface.cgi](http://greengenes.lbl.gov/cgi-bin/nph-blast_interface.cgi)).

### **Statistical analysis of next generation sequencing**

The relative abundance of OTUs were normalized by the total cell count within each sample then analysed for diversity index (Shannon and Simpson) with statistical

significance by performing t-tests ( $p$ -values at 0.05) using SPSS version 20. The values from plaque samples collected from the healthy group were compared to those from the periodontal disease group using R software and heatmaps were constructed using R package gplots (Warnes et al. 2014). The relations of microbial community data were produced by PERMANOVA for significant differences (P value) and size of effect (F. model) and illustrated as principal component analysis plots (PCA) using R package vegan (Dixon 2003).

## Results

### Culture dependent (or phenotypic) identification of bacteria

The results of 53 isolates from the healthy group and 63 isolates from the periodontitis group are shown in Table 4 and represent a range of species from a number of genera (healthy = 15, periodontitis = 16 genera). The most common isolates found were *Prevotella* sp. (13% include *P. dentasini* 6.1%, *P. intermedia* 5.2%), *Porphyromonas* sp. (9.6% include *P. asaccharolytica* 3.5%, *P. uenonis* 1.7%), *Staphylococcus* (8.7%), *Streptococcus* (7.8%), *Actinomyces* (6.1%), *Fusobacterium* sp. (5.2% include *F. equinum* 1.7%, *F. nucleatum* 1%) and a number of isolates were identified by Genbank as “uncultured” species/clones (17.4%).

**Table 4** Bacterial species, grouped as families, identified by 16S rRNA gene sequencing obtained from four horses with normal periodontal tissues/gingivitis and four horses with periodontitis grouped as families.

Phylum	Species [% identity range]	Health/gingivitis No. of isolates (% of total) n = 53	Periodontitis No. of isolates (% of total) n = 62
<u>Actinobacteria</u>	<i>Actinomyces denticolens</i> [98]	1 (0.9)	
	<i>Actinomyces massiliensis</i> [95]		1(0.9)
	<i>Actinomyces suimastitidis</i> [95]		1(0.9)
	<i>Actinomyces urogenitalis</i> [96]		1(0.9)
	<i>Actinomyces viscosus</i> [91]		1(0.9)
	<i>Actinomyces</i> spp. <sup>1</sup> [98]	2(1.7)	
	<i>Atopobium minutum</i> [98]	1(0.9)	
	<i>Atopobium</i> spp. <sup>1</sup> [85]	1(0.9)	
	<i>Olsenella profusa</i> [90]		2(1.7)
	<i>Olsenella</i> spp. <sup>1</sup> [97]		1(0.9)
	<i>Parascardovia denticolens</i> [98]		1(0.9)
	<i>Propionibacterium acnes</i> [99]		1(0.9)
	<i>Propionibacterium</i> spp. <sup>1</sup> [98-99]	1(0.9)	1(0.9)
	<i>Slackia exigua</i> [94]	1(0.9)	
	Uncultured <i>Actinobacterium</i> spp [97]		1(0.9)

<u>Bacteroidetes</u>	<i>Porphyromonas asaccharolytica</i> [87-90]	2(1.7)	2(1.7)
	<i>Porphyromonas macacae</i> [91-99]	2(1.7)	2(1.7)
	<i>Porphyromonas uenonis</i> [92-96]		2(1.7)
	<i>Porphyromonas</i> spp. <sup>1</sup> [93]		1(0.9)
	<i>Prevotella dentasini</i> [78-99]	3(2.6)	4(3.5)
	<i>Prevotella jejuni</i> [95]		1(0.9)
	<i>Prevotella intermedia</i> [98]	4(3.5)	2(1.7)
	<i>Prevotella</i> spp. <sup>1</sup> [91]	1(0.9)	
<u>Firmicutes</u>	<i>Filifactor villosus</i> [99]		1(0.9)
	<i>Lachnoanaerobaculum umeaense</i> [93-94]	1(0.9)	1(0.9)
	<i>Lactobacillus alvi</i> [98]		1(0.9)
	<i>Lactobacillus</i> spp. <sup>1</sup> [98]		1(0.9)
	<i>Parvimonas</i> spp. <sup>1</sup> [97]	1(0.9)	
	<i>Peptostreptococcus anaerobius</i> [86]	1(0.9)	
	<i>Sharpea azabuensis</i> [99]		1(0.9)
	<i>Staphylococcus aureus</i> [98-99]	3(2.6)	
	<i>Staphylococcus epidermidis</i> [97-99]	1(0.9)	3(2.6)
	<i>Staphylococcus pasteurii</i> [99]		1(0.9)
	Uncultured <i>Staphylococcus</i> spp. [98]	1(0.9)	
	<i>Streptococcus criceti</i> [96-99]	1(0.9)	3(2.6)
	<i>Streptococcus dentasini</i> [99]	1(0.9)	

	<i>Streptococcus</i> spp. <sup>1</sup> [83-98]	3(2.6)	3(2.6)
	<i>Veillonella atypica</i> [93]		2(1.7)
	<i>Veillonella</i> spp. <sup>1</sup> [94-96]	2(1.7)	1(0.9)
	<i>Eubacterium</i> spp. <sup>1</sup> [86-91]		3(2.6)
Fusobacteria	<i>Fusobacterium equinum</i> [96-97]	1(0.9)	1(0.9)
	<i>Fusobacterium nucleatum</i> [96]	1(0.9)	
	<i>Fusobacterium</i> spp. <sup>1</sup> [97-98]	1(0.9)	2(1.7)
<u>Proteobacteria</u>	<i>Citrobacter</i> spp. <sup>1</sup> [88]	1(0.9)	1(0.9)
	<i>Enterobacter</i> spp. <sup>1</sup> [74]	1(0.9)	1(0.9)
	<i>Actinobacillus equuli</i> [99]	1(0.9)	
	<i>Actinobacillus lignieresii</i> [97]	1(0.9)	
	<i>Campylobacter curvus</i> [90-91]	2(1.7)	
	<i>Campylobacter gracilis</i> [96]		1(0.9)
	<i>Campylobacter showae</i> [85]		1(0.9)
	<i>Campylobacter</i> spp. <sup>1</sup> [96]	1(0.9)	
Uncultured	Uncultured bacterium <sup>2</sup> [86-99]	7(6.1)	9(7.8)
	Uncultured methanogenic archaeon [98]	2(1.7)	

<sup>1</sup> unable to distinguish between species; <sup>2</sup> represent a number of species as defined by phenotypic tests



### Next generation sequencing method

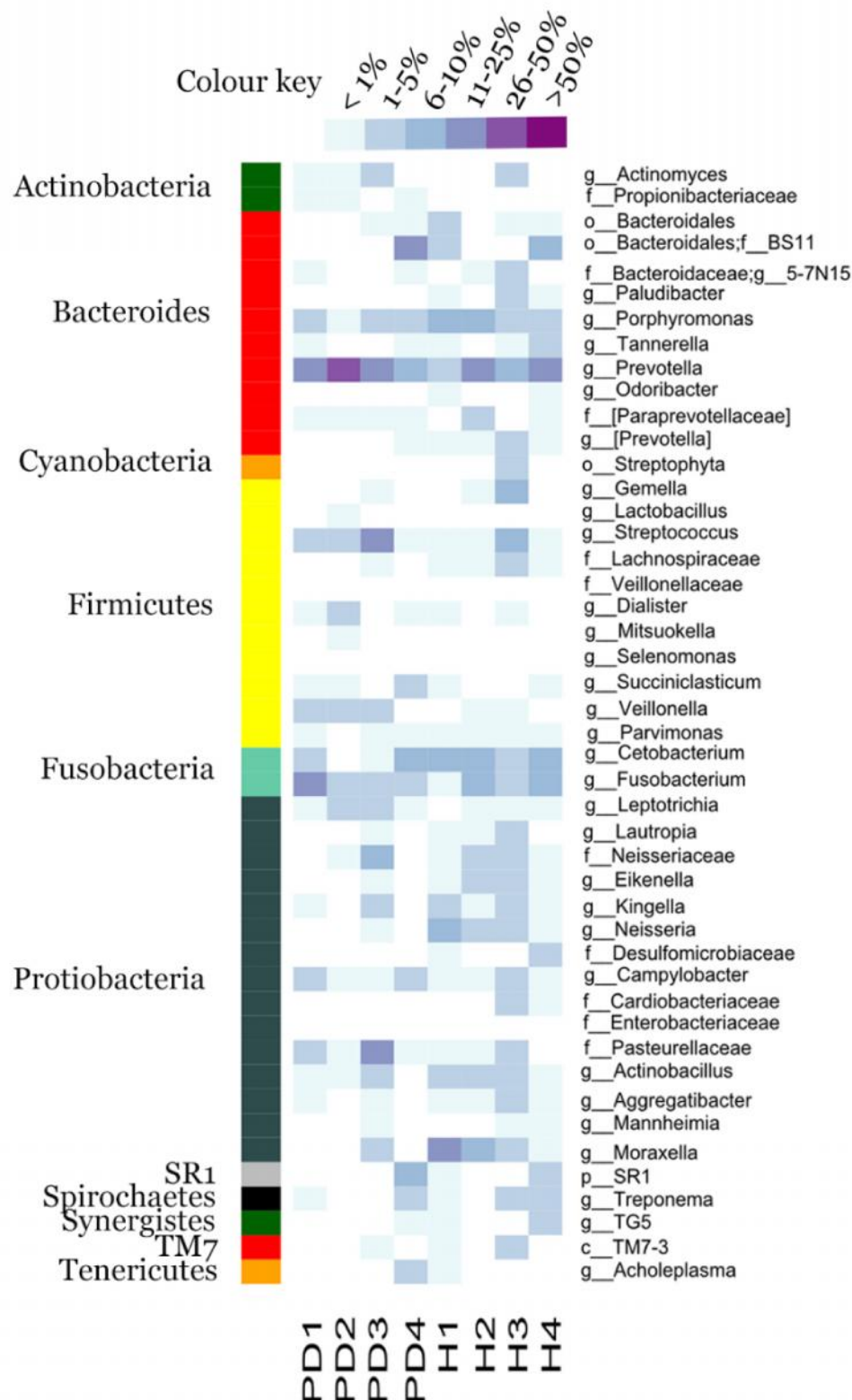
Sequencing generated a total of 1,272,289 reads for all eight samples (mean 159,036 reads per samples). After removing sequence errors and cleaning using R Studio, the dataset decreased by approximately 53.5% to a total of 592,019 sequences with average 85,033 sequences per sample, (63,990 – 133432; standard deviation 22,314).

Across all samples, sequences were clustered into 203 OTUs, representing 11 different bacterial phyla. Only 46 OTUs presented with a relative abundance of at least 1%. The relative distribution/HEATMAPS of the OTUs at genus level with abundance of at least 1% are illustrated in Figure 7 and at phylum level in Figure 8. Shannon and Simpson diversity indexes and OTUs numbers are presented in Table 5, and showed no statistical significance between the two groups. Data were visualized using PCA plots describing dissimilarity (Figure 9).

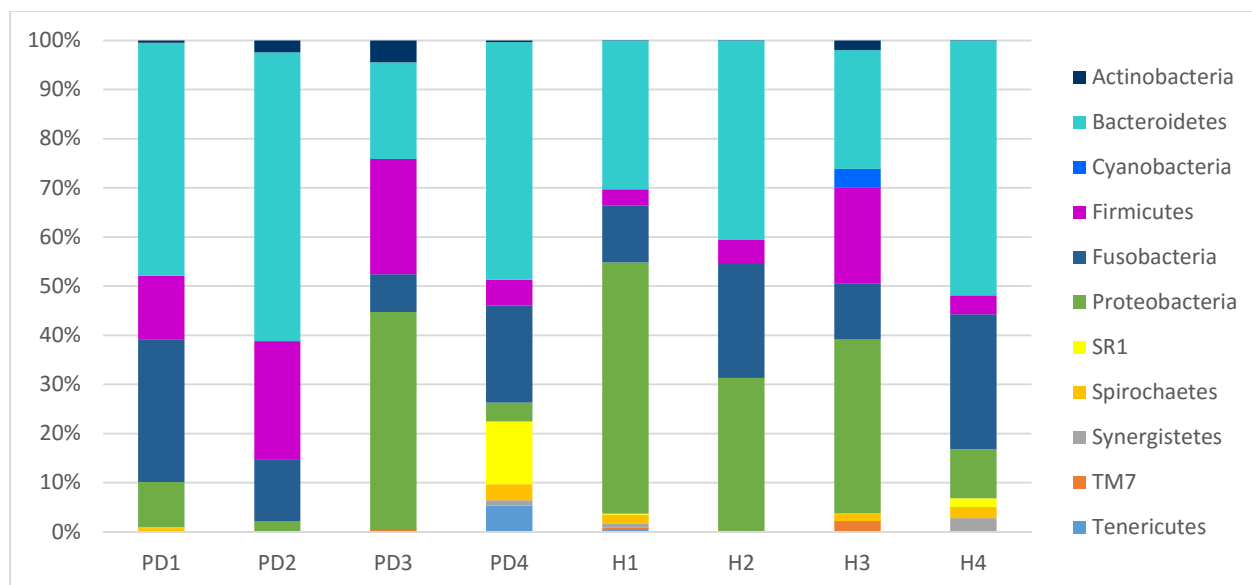
These results at the phylum-level and a previous study that used culture dependent methods (Bailey & Love 1991) showed the same result that Firmicutes has the highest prevalence in samples taken from pharyngeal tonsillar and para oral infections (Figure 10).

**Table 5** Diversity statistics of bacterial community from 8 horses with different stage of periodontal disease as determined by next generation sequencing, (PD = periodontal disease, H = healthy).

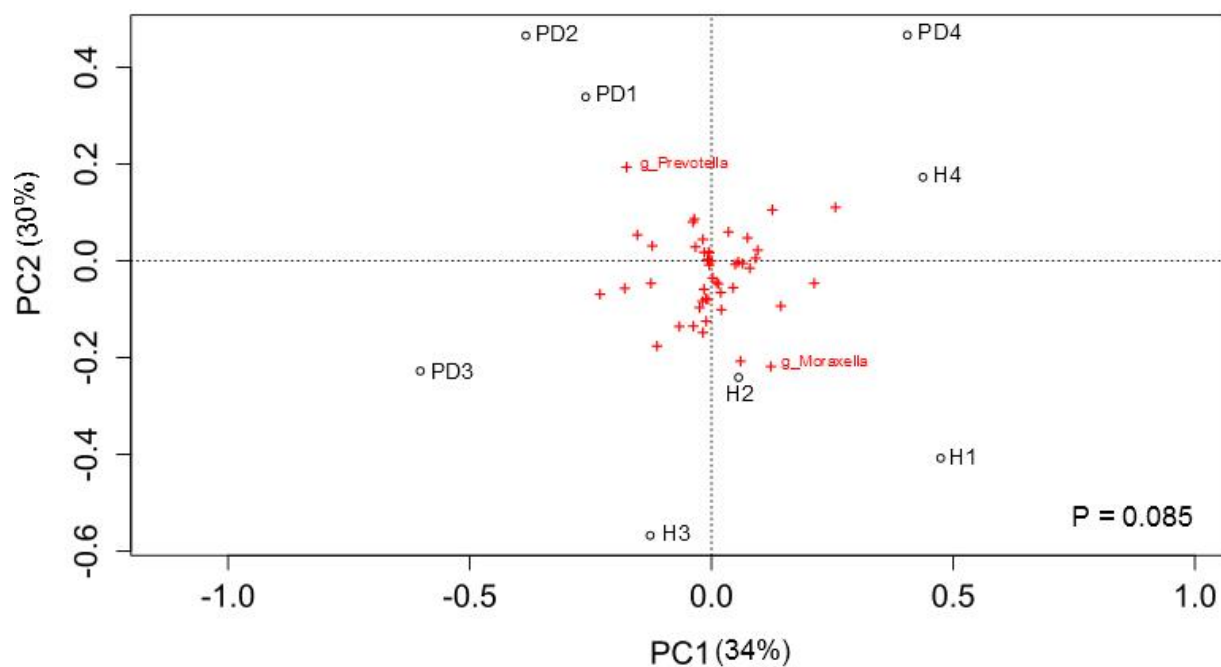
	PD1	PD2	PD3	PD4	H1	H2	H3	H4
Total sequences	122757	116355	178022	137592	186145	135686	133155	262577
No. OTUs	137	129	146	123	134	115	147	131
Simpson	0.76087	0.66045	0.87796	0.88625	0.87477	0.88426	0.95015	0.88307
Shannon	1.98528	1.77071	2.43757	2.52849	2.62502	2.50195	3.21772	2.64147



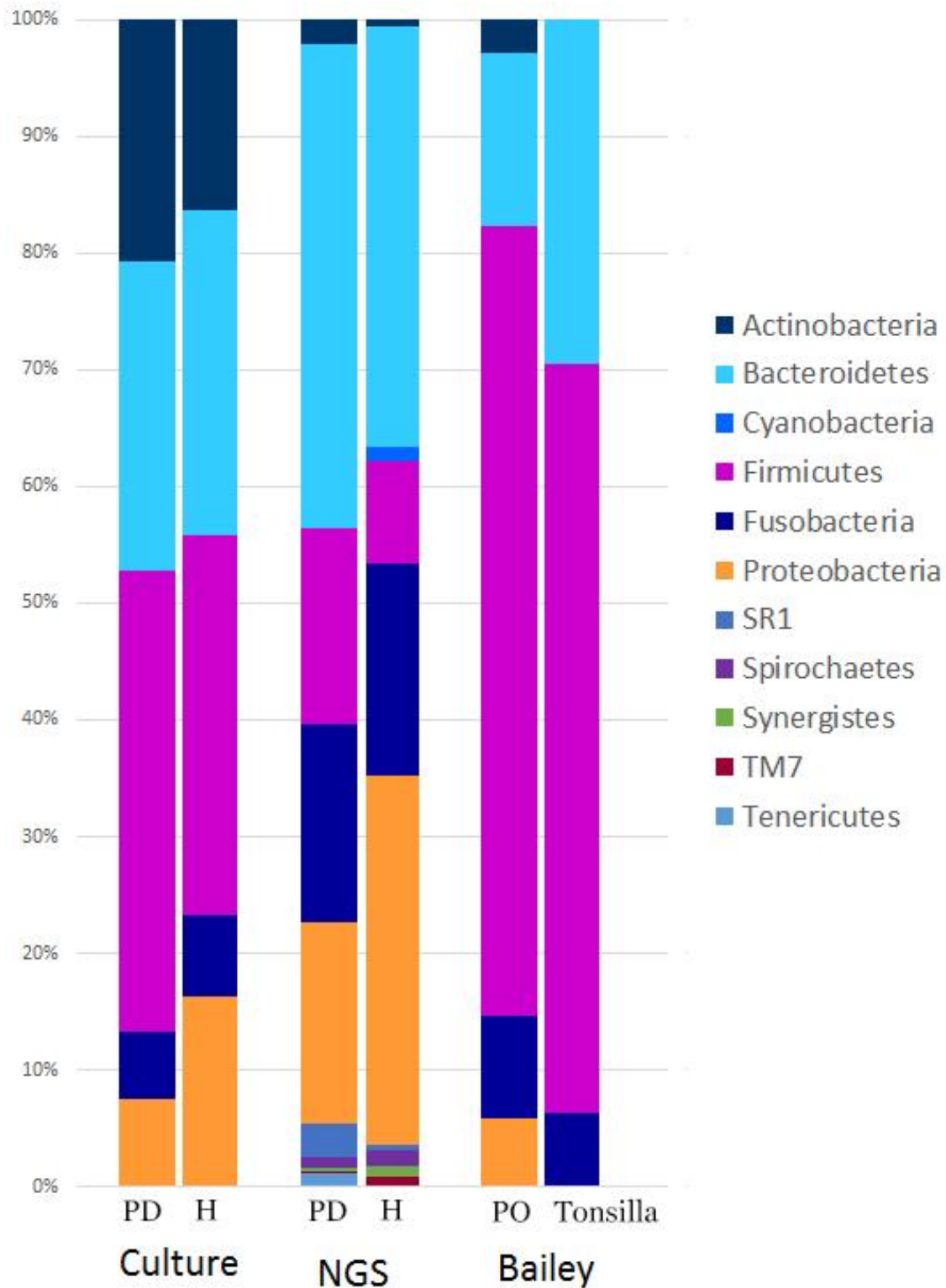
**Figure 7** Genus-level relative abundance of eight plaque samples collected from horses. Taxa shown are at genus-level (g\_) or at next lowest taxonomic classification as indicated, PD = periodontitis group, H =healthy group.



**Figure 8** Relative abundance of bacteria phyla in the oral cavity of horses with different periodontal disease status, PD = periodontitis group, H = healthy group.



**Figure 9** Principle component analysis of horse plaque samples with next generation sequencing. P value calculated using PERMANOVA.



**Figure 10** Phylum level comparison of horse oral microbial community using culture dependent and NGS methods with culture results adapted from the study of Bailey & Love 1991 (Culture, NGS and Bailey, respectively). PD = periodontitis group, H = healthy group, PO = para oral infection, Tonsilla = pharyngeal tonsillar collection.

## Discussion

Equine periodontal disease is one of the most common inflammatory diseases of the equine oral cavity. The results from this part of the thesis on the oral microbiology of horses with health or periodontitis shows that the microbiome is highly diverse. This is the first study to use combined NGS and culture dependent method to identify bacterial isolates in the equine oral cavity. The results also demonstrate the benefits of molecular methods, such as next generation sequencing, to extend our knowledge of the diversity of bacterial species involved in both the healthy equine oral cavity and in horses with PD.

We have observed total of 16 genera in both healthy and diseased groups. The most prevalent genera found was *Prevotella* and differences were seen between the groups: 15 species were only found in healthy group and 20 were found in only periodontitis group. Black pigmented anaerobic bacteria such *Prevotella* and *Porphyromonas* have been isolated from the oral cavity of horses with several new species recently discovered (Mackintosh & Colles 1987; Bailey & Love 1991; Takada et al. 2010). The confirmation of identification to species-level using 16S rRNA sequencing of bacterial isolates was shown to be the best approach. In this study two organisms (*Actinobacillus equuli* and *Actinobacillus lignieresii*) were cultured from the healthy group that have been reported in human infections associated with horse bites, (Moore et al. 2003). These isolates have the potential to cause respiratory infections and bite wounds in humans, especially equine veterinary personnel who have close contact with horses.

A number of isolates were identified from the NCBI taxonomic database (GenBank®) with less than 97% identity match with the 16S rRNA sequences and reported as “uncultured/clones” or unclassified at the species-level, highlighting the high potential for the presence of novel species (Stackebrandt & Goebel 1994). These isolates included those from genus *Porphyromonas* (90%, 16S rRNA sequence identity match) and *Lachnoanaerobaculum* (93-94%, 16S rRNA sequence identity match). However, further work, such as a complete 16S rRNA gene sequencing and comprehensive phenotypic characterisation are needed to confirm that these species are novel.

There was no statistical difference between the diversity indexes of OTUs between both groups. The predominating phyla in the periodontitis group was Bacteroidetes and three phyla (Firmicutes, Fusobacteria and Proteobacteria) which had similar prevalence. In the healthy group, the community was dominated by Bacteroidetes and Proteobacteria. The microbial community at phylum-level was consistent with results reported in a similar periodontal study on six healthy dogs (Sturgeon et al. 2013) but in contrast with a study on 11 healthy cats where the majority of periodontal phyla was Proteobacteria (Sturgeon et al. 2014).

The minority phyla included SR1, Spirochaetes, Synergistetes, and TM7 which were in low abundance in both the periodontitis and healthy groups (3.6% and 5.3%, respectively). At the genus-level, the equine bacterial community was dominated by *Prevotella* with higher prevalence in the periodontitis group than healthy group but no statistical difference ( $P < 0.5$ ), as illustrated in the heatmaps. *Prevotella* was also reported in the studies of dogs (Sturgeon et al. 2013) and cats (Sturgeon et al. 2014). Each of the equine oral bacterial genera presented with a relative abundance less than 25% in all samples, except *Prevotella* that presented with higher prevalence in all samples.

PCA using the Hellinger method reduced the effect of dominant populations in the samples and allowed more meaningful interpretation of microbial ecology (Legendre & Gallagher 2001). At the genus-level OTUs were clustered around the centre origin. Metadata were also correlated with the sequencing results using PERMANOVA for statistical analysis of multivariate data sets (Dixon 2003). There was low correlation between the sequencing data and periodontal status of samples and it was not statistically significant ( $P < 0.1$ ). This means that the periodontitis and healthy groups share similar bacterial community members.

NGS yielded dramatically different results than conventional culture methods. Comparing the number of isolated bacterial species (55 species in total) to the overall number of OTUs (203 total) obtained from NGS showed that only one fourth of the bacterial community can be isolated using the culture methods adopted in this study. This may result from bacteria not surviving transport or the need for special requirements for growth. On the other hand, cultivation of bacteria allowed phenotypic confirmation of the 16S rRNA sequencing results. Combining the cultivation of isolated

bacteria with molecular methods enables novel bacteria to be accurately identified and described. In addition, antibiotic sensitivity testing requires cultivation to assess chemotherapeutic treatment efficacy.

Despite the high number of recovered bacteria identified, NGS Illumina software was not able to provide species-level identification. However, a broader range of community profiling provided greater information at the genus-level. It is anticipated that this situation will improve with submission of sequences of isolates to databases from the studies of oral cavities of animals.

Prior to this study, it was hypothesized that equine oral bacterial populations will differ between healthy and periodontitis groups, similar to that observed in the oral cavity of dogs (Riggio et al. 2011) and cats (Booij-Vrieling et al. 2010). The results, however, showed a similar prevalence of bacterial species in both studied groups. These differences between animal species may be due to differences in dentition and eruption, feed type, and husbandry. There may be novel bacteria that are only found within each oral cavity of these animals. Despite this, there is the similarity of relative abundance at the phylum-level of the study conducted in this thesis to previous a study of horses (Bailey & Love 1991). However, the result from NGS shows that there are differences in relative abundance of equine oral bacteria. Firmicutes share a smaller proportion compared to Bacteroidetes and Fusobacteria. The rest of phyla could not be identified using culture dependent methods. This may due to the limitation in culture methods and media that were used in this study.

### **Conclusions and Limitations of study**

Despite the small number of samples involved in this study, a wide range of bacteria were detected within the healthy and periodontitis groups with similar prevalence of species in both groups. This result differs from studies of periodontal disease in other domestic animal species, perhaps due to the continued eruption of equine teeth which allows for regeneration of periodontium if the ongoing inflammation due to bacteria is managed. Moreover, we could not relate horse age to bacterial community species. This area may be clarified by future large scale studies. Culture dependent techniques provided deeper identification to the species-level, although a

smaller range of bacteria were recovered compared to NGS, which could mainly identify to the genus-level.

Although, the results of this study have substantially improved our understanding of the equine oral microbiome, there are many limitations that need to be considered. Firstly, culture dependent methods where each bacterium may have different growth requirements can result in a limited number of isolates. Secondly, molecular methods employing NGS could only identify to the genus-level, which provides an overview of the bacterial communities but not to the species-level. Other molecular methods such as cloning of 16S rRNA PCR products would more likely identify species (Riggio et al. 2011). Thirdly, increasing the numbers of animals in a future study with larger numbers of samples from differing horse ages would provide a more complete picture of the equine oral microbiome.



## **Chapter 5:**

**The use of intra-oral radiography and 3D reconstructed image from computed tomography for diagnosis of periodontal disease in equine cheek teeth.**

## **Introduction**

The basic assessment of periodontal status in animals such as dogs and cats was adapted from humans and used measurement of the periodontal pocket depth and finding of furcation exposure where severe alveolar bone loss is present. Klugh (2005a) described a method of determining the stage of periodontal disease in horses that was adapted from the human dentistry protocol, using percent attachment lost and a mobility index. However, using probing depth as the sole criteria will not provide a true indication of the extent of periodontal disease as the length of the reserve crown in a particular horse is unknown by visualisation and/or palpation examination. Therefore a fixed term measurement for grading of EPD, such as physical measuring of probing depth using a periodontal probe, is unreliable. The use of radiographic imaging of the coronal region of the tooth for EPD assessment is essential.

The normal anatomical, histological and micro anatomy of equine dental structures and periodontium has been extensively studied by German and Irish research groups (Staszyk & Gasse 2004a; Masset et al. 2006a; Staszyk et al. 2006; Cox et al. 2012) studies of radiographic assessment of equine periodontal disease are limited. There have been reports describing techniques for intraoral radiography in horses (O'Brien 1996; Klugh 2005f; Baratt 2013). However, these techniques and equipment were before the development of dedicated equine intraoral computed radiography (IO-CR).

### **Equine IO-CR**

Intraoral radiography has been in use for equine dentistry for many years but mainly by using wet preparation film, which involved chemical risks (Klugh 2005f; Baratt 2013). Recently, a Swedish research group (Uhlhorn et al. 2008) successfully used a specially modified CR plate (4-7×12cm), to evaluate specific teeth and reported that the image plate was easy to use and handle. The advantages of IO-CR are the elimination of superimposition of the opposite tooth arcade and the reduction of distortion of the tooth shape by applying the bisecting angle technique to the projection (Klugh 2005f; Rubin et al. 2008).

### **3D imaging in equine dentistry**

CT techniques are rapidly expanding and are becoming a more routine diagnostic tool in veterinary science. The advantages of CT in comparison to IO-CR for periodontal disease diagnosis are the increased sensitivity of tissue differentiation because a thin cross sectional “slice” of the structure can be imaged at a high resolution. Bone and soft tissue relationships can be examined using the modern technology of 3D image reconstruction (Barbee et al. 1987; Barbee & Allen 1990). In a mouse model of periodontal disease, quantitative measurement of periodontal ligament and alveolar bone loss can be performed using micro-CT, which can provide a higher resolution image than medical or dental clinical CT (Park et al. 2013). This model of quantitative measurement of periodontal tissue could be adapted to the equine cheek teeth model on CT imaging. A recent study on equine sinus structure and cementum of equine incisor using 3D CT imaging showed that it can provide more detailed information than standard 2D cross sectional images (Brinkschulte et al. 2014, Schrock et al. 2014). The 3D may be useful for some advanced clinical situations, research and education. However, cost, special skill requirements, time consumption and risk of anaesthesia are still considered to be major limitations for everyday use.

The purpose of this study was not to compare IO-CR and 3D imaging, but was conducted to describe and evaluate periodontal lesions of equine cheek teeth that could be identified by these methods.

### **Materials and methods**

All protocols for this project were approved by the Animal ethics committee of the University of Queensland (No. ANRFA/SVS/331/13). All the procedures were carried out at the School of Veterinary Science University of Queensland, Gatton.

#### **Specimen selection, clinical assessment and periodontal assessment**

Horse heads in this study were acquired from an abattoir at Caboolture, Queensland, Australia, where horses are commercially slaughtered. As part of the processing at the abattoir, all heads are skinned and the tongues and the masseter muscles removed. The heads were kept frozen with the mouth open then thawed in

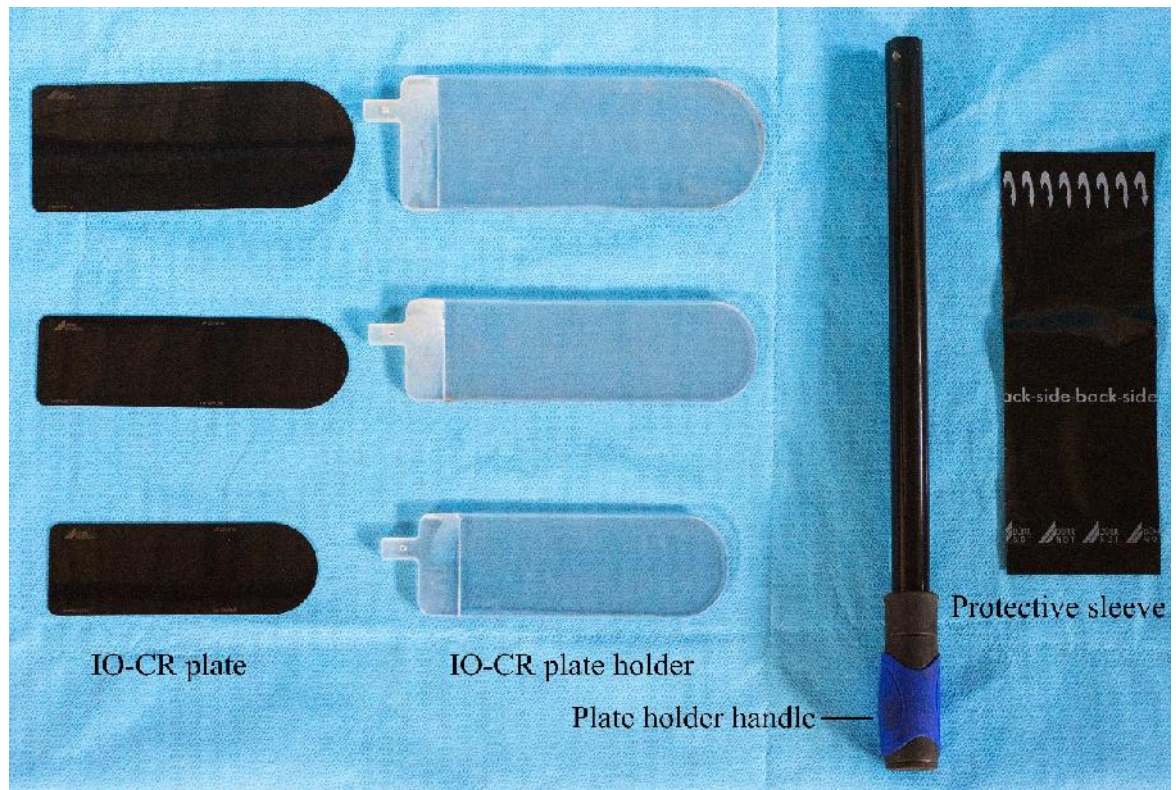
the cold room one day before the examination. Routine dental examinations were performed prior to the radiographic study, using visual inspection and palpation for EPD with auxiliary tools such as a dental mirror, dental pick and periodontal forceps. All periodontal pockets (PP) where depth was greater than 5 mm and the presence of diastema (DI), as described by Dixon and Dacre (2005), were recorded on a dental chart. Heads with at least one of the above lesions (PP and DI) were selected and a head without any lesions, as the control were selected.

### **Intraoral computed radiography protocol and positioning**

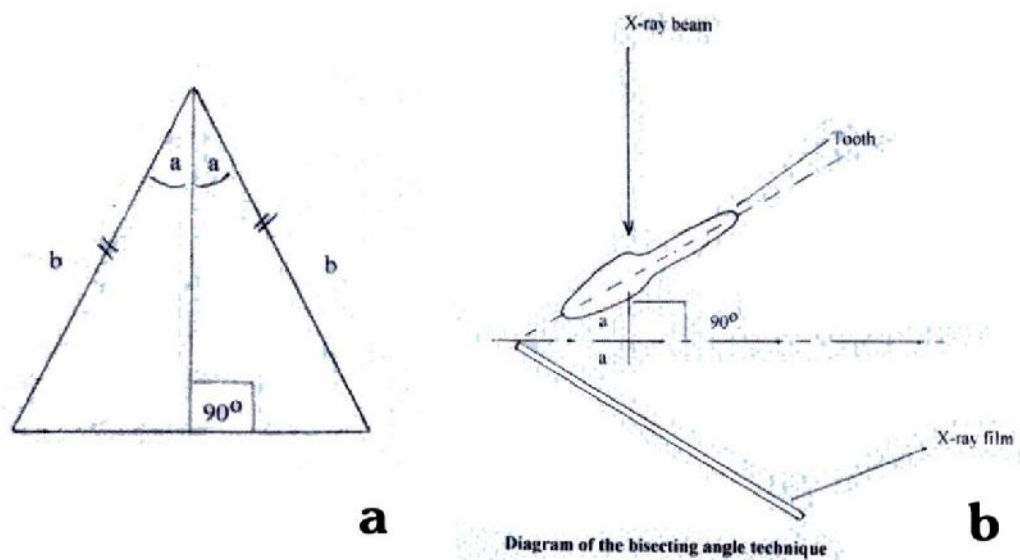
The radiographic images were taken at the School of Veterinary Science University of Queensland Gatton Campus. Horse heads were radiographed with an X-ray portable beam generator (Poskom Vet-20BT, Goyang, Korea) and photostimulable phosphor plates (PSP) then scanned with a CR35 VETwin® scanner (iM3 Pty Ltd New South Wales, Australia) as shown in Figure 11. Two main positions as described below were applied to all specimens. Other additional intraoral positioning was used if required.

### **IO-CR positioning and beam direction for maxilla cheek teeth**

In order to avoid superimposition of adjacent teeth, the bisectational angle technique was applied as follows: the X-ray beam (76 kVp, 20 mAs) was directed at the tooth of interest perpendicular to an imaginary line that bisected the angle between the tooth and intraoral cassette as shown in Figure 12. By applying this technique to the specimen, the intraoral cassette with IO-CR plate was placed parallel to the occlusal surface of the maxillary cheek teeth and approximately parallel to the hard palate, and the X-ray beam was aimed parallel to the interproximal angle of the tooth of interest and the adjacent tooth as shown in Figure 13.

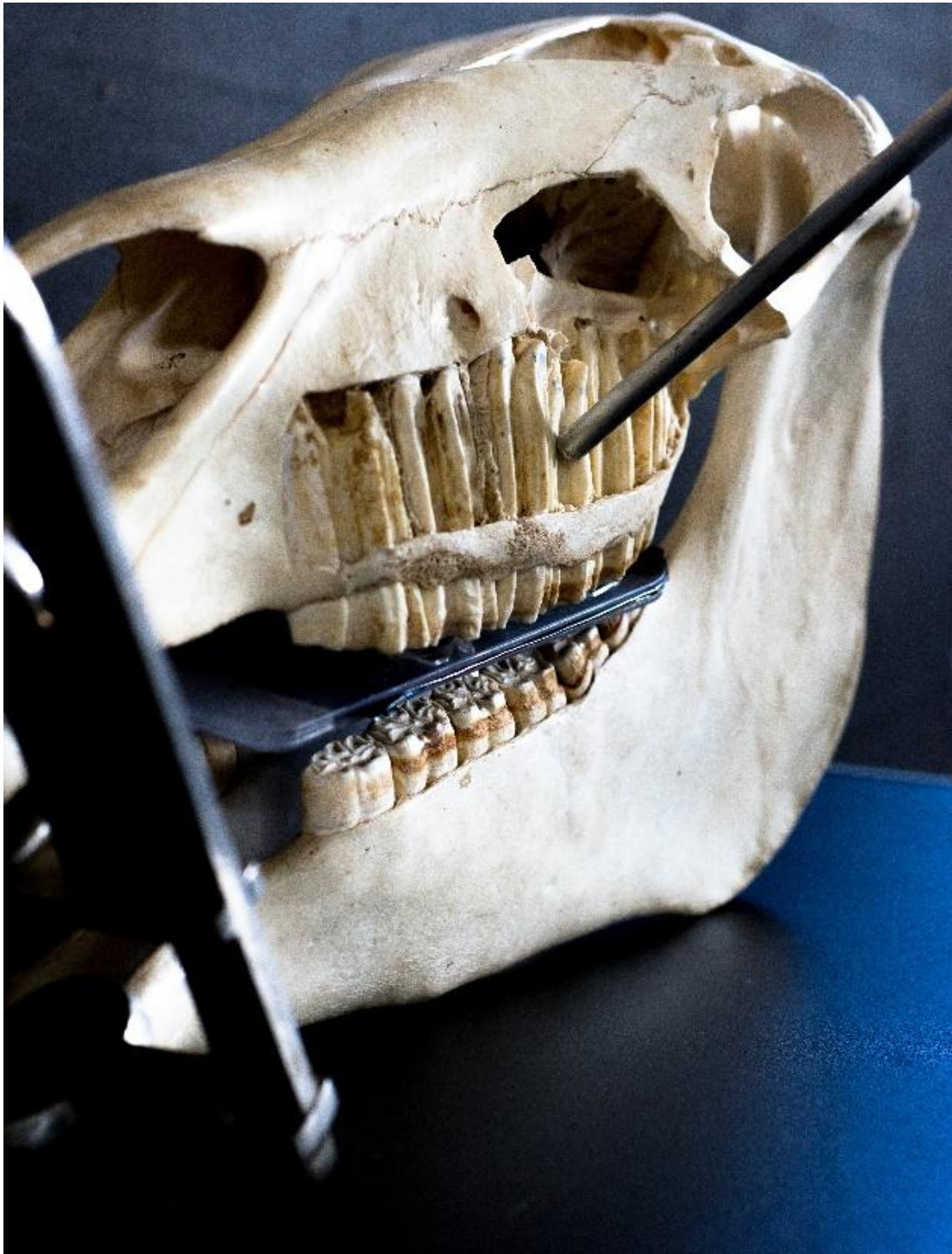


**Figure 11** Intraoral computed radiography plate, plate holder, plate holder handle and protective sleeve.



**Figure 12** Bisecting angle technique (photo courtesy from Gary J Wilson, School of Veterinary Science University of Queensland, Australia).





**Figure 13** Intraoral plate (black plate) positioning and X-ray beam (aluminium rod) direction on dry skull for maxillary cheek teeth.

## **IO-CR positioning and beam direction for imaging the coronal region of mandibular cheek teeth**

The intraoral plate was placed parallel to the long axis of the cheek teeth arcade. The X-ray beam (70 kVs, 2.0 mAs) was aimed at the tooth of interest and directed perpendicular to the tooth. The X-ray beam was also parallel to the interproximal angle of the tooth of interest and adjacent tooth in order to avoid superimposition.

## **Computed tomography protocol and post-processing of digital imaging and communications in medicine (DICOM) data**

All of the specimens were scanned at the Equine Hospital, University of Queensland Veterinary Medical Centre, Gatton. All heads were imaged by a third generation CT unit (Toshiba Activion 16 scanner, Toshiba (Australia) Pty LTD, New South Wales Australia). The frozen heads were placed on the patient table in ventrodorsal position. Scanning commenced at the incisal edge of the incisors and continued caudally, ending at the caudal edge of the vertical rami of the mandible. The following parameters were used: 135 KV, 250 mA and 1 mm slice thickness. The DICOM data set was investigated using the OsiriX image processing software (Rosset et al. 2004) and reconstructed a 3D image with automated function “3D viewing, 16-bit CLUT”. In addition, clinical periodontal attachment level (AL) of the periodontal ligament was measured and used to calculate percentage 2D CT images where periodontal lesion using following equation.

$$\frac{\text{distance from estimated highest point of crestal bone to end point of periodontal lesion}}{\text{distance from estimate highest point of crestal bone to root apex}} \times 100$$

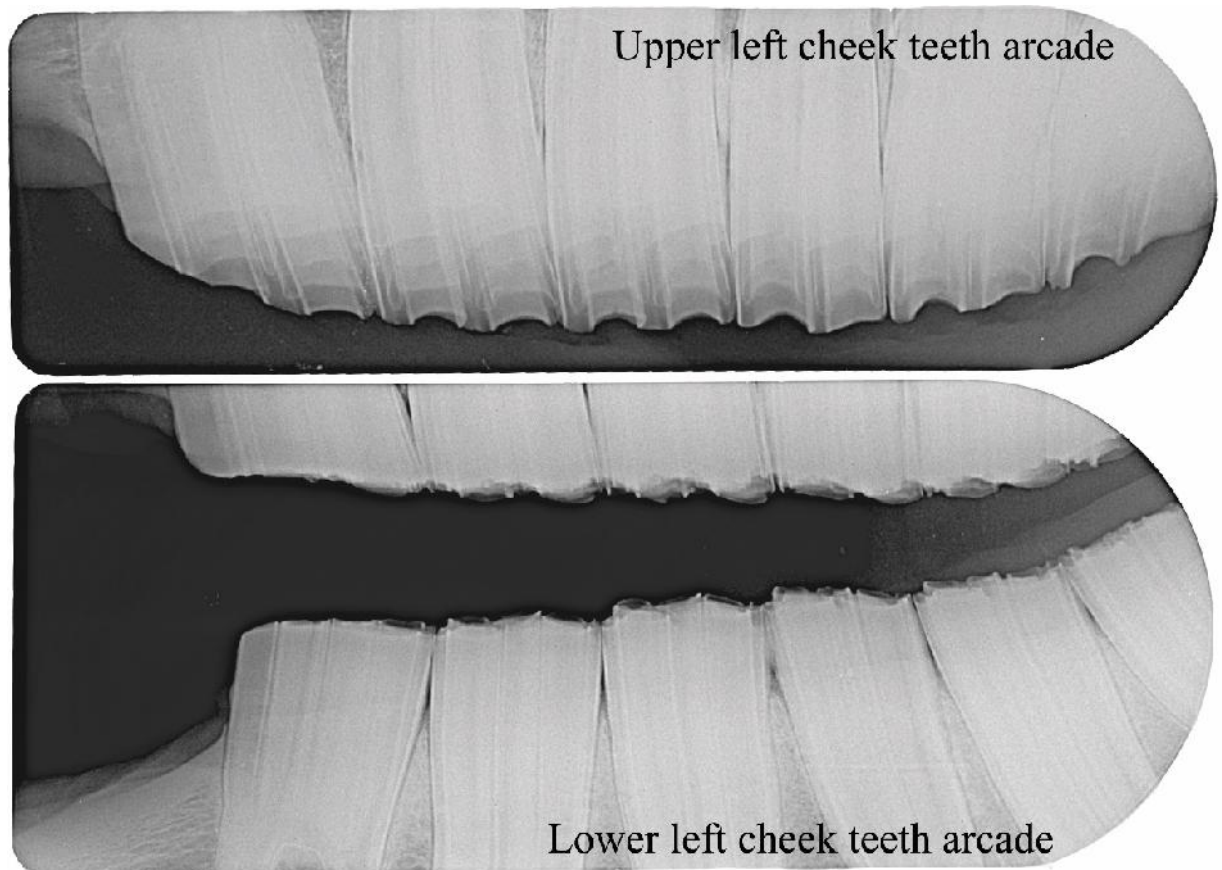
For example: calculation alveolar bone on rostral aspect of 209 of horse H-04 (Figure 20)

$$\frac{0.901}{2.277} \times 100 = 39.6\%$$

## **Interpretation of IO-CR images and 3D reconstructed images**

All radiographic lesions of periodontal disease from IO-CR and 3D reconstructed images included widening of the periodontal ligament space (WS), PP, DI, reduced crestal bone height (CB), inter radicular bone loss/furcation lesion (FL), and alveolar

bone loss (AL). The details and description of lesions and tooth identification are described in previous studies (Klugh 2005f; Floyd 1991). Lesions were described and compared with control images acquired from an 8 year old horse presented with smooth, clear periodontal ligament space and good crestal bone on radiographic images as shown in Figure 14.



**Figure 14** Intraoral radiographic image of an 8 year old horse with no radiographic evidence of PD.

## Results

Due to the protocol and safety concerns for visitors at the abattoir, the examiner was not allowed access to the holding area to examine the live horses for gender and general health. A total of seven horse heads were aged, examined, radiographed and CT scanned. Six heads had one or more clinical signs of EPD (13-20 years of age, with an average age of 15 years), one head (H-05, age 8 years old) had no clinical nor radiographic evidence of periodontal disease, which was used as the control. Details



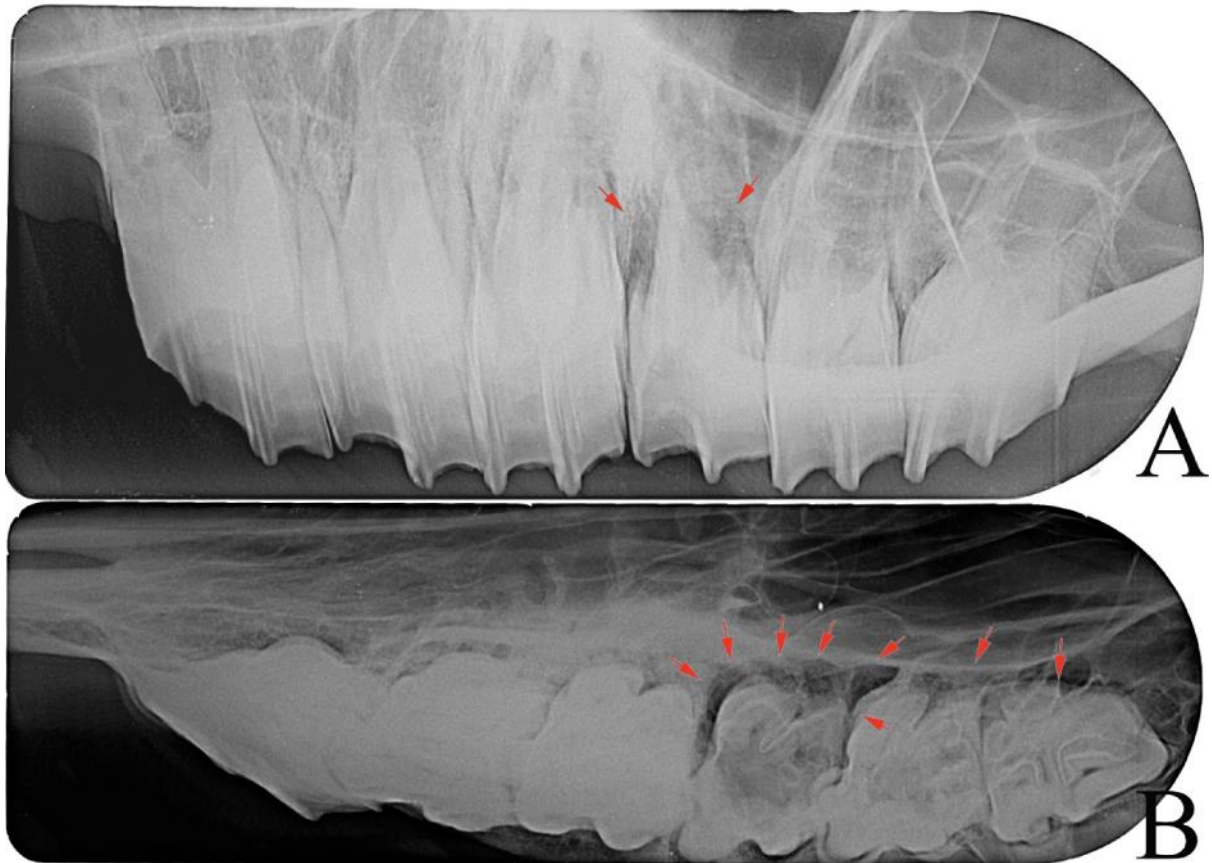
of radiographic evidence from IO-CR, CT and 3D imaging are shown in Table 6. The average time required for a CT scan was 40 seconds with additional time of 40-50 minutes for DICOM file transfer and 3D image reconstruction, whereas an average of 45 minutes was required to obtain a completed 4-6 views of IO-CR, including positioning and plate scanning. The longer time required for a CT scan did not include patient preparation, anaesthesia and recovery time, which may have increased the time up to one or two hours.

### **Image quality and periodontal lesion**

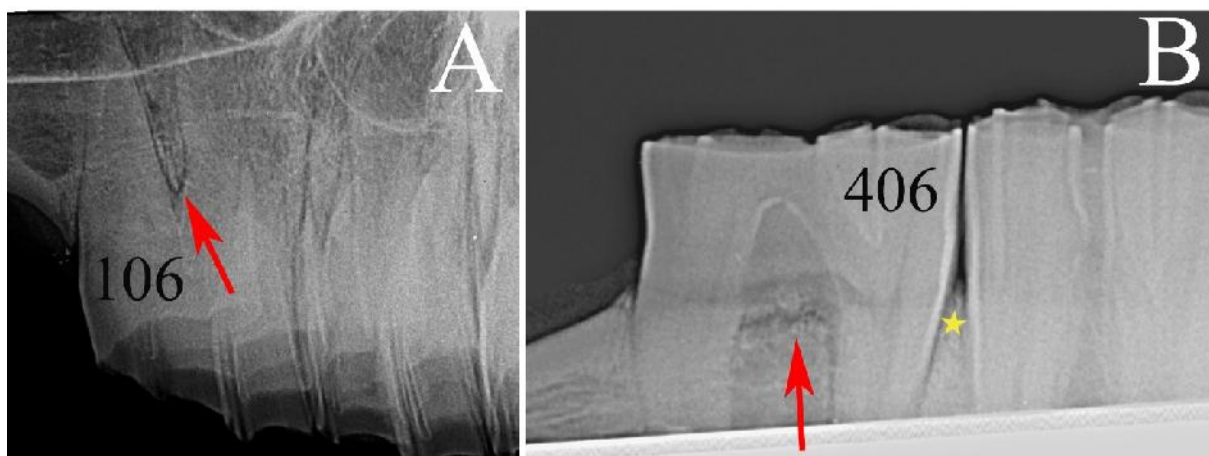
In this study IO-CR demonstrated good diagnostic quality images of a single dental arcade, whereas 3D demonstrated bone and dental structure of the whole of the scanned areas in a single 3D image.

Both IO-CR and 3D imaging allowed the accurate diagnosis of periodontal disease in all the cases whereas on oral examination some were not detected. IO-CR also demonstrated the detailed structure of the periodontium, such as lamina dura, crestal bone and tooth structure. However, a simple 3D image only showed the external structure of the scanned area which can be manipulated for internal structure with more complex programming.

A complete set of standard views with an additional intraoral dorso-ventral view in an 18 year old horse with severe periodontal disease around the 109 allowed visualisation of the complete destruction of the periodontium (Figure 15a and Figure 15b) and inter-radicular bone loss (Figure 16). On the other hand, 3D imaging shows an image of bone and tooth structure not impeded by soft tissue, such as in Figure 17 and Figure 18 compared to the normal view from oral examination Figure 19. In addition, 2D images from a CT file allows AL evaluation and attachment loss, as illustrated in Figure 20 with the results included in Table 6.



**Figure 15** IO-CR images from an 18 year old horse (H-07) with periodontal lesions around the 109: (a) standard oblique projection for maxilla arcade; (b) additional intraoral dorso-ventral view. Number and red arrows indicate tooth nomenclature and pocket margin.



**Figure 16** IO-CR images from an 18 year old horse (H-09) with interradicular bone loss lesion at (a) 106, (b) 406). Number, red arrows and yellow star indicate tooth nomenclature, furcation lesion and DI respectively.

**Table 6** Description of diagnostic and periodontal abnormalities of cheek teeth for comparison between intraoral computed radiography (IO-CR) and 3 dimensional (3D) imaging.

Horse ID	Dental age (years)	Oral examination finding	IO-CR findings	3D findings with percentage of attachment loss (%)
H-5/control	8	Visibly healthy gum.	None	None
H-03	18	Open valve DI between mandibular cheek teeth.	FL lesion at 106, 206, 306, 406 and 407. WS, lesion apically of 109 and AL apically at 209. CB present at most of mandibular teeth.	PP, palatal AL around 109 (32%) and 209 (51.1%). No visible lesion on mandible arcades.
H-04	17	PP between lingual aspects of 407-408.	WS and AL apically of 109. FL lesion at 306 and 406. CB between 306-307 and 308- 309.	Palatal AL at rostral of 109 (20.7%). Buccal AL around 108 (27.5%) and 209 (39.6%). No visible lesion of mandible arcades.

Abbreviation: DI = diastema; FL = inter radicular bone loss/furcation lesion; WS = widen periodontal ligament space; AL = alveolar bone loss; CB = reduced crestal bone height; PP = periodontal pocket.

H-06	13	DI present all mandible cheek teeth.	WS apically at 107. CB PP and AL between 208-209 apically of 208. DI present (25.2%). Visible FL at 306 (0%) and between all left mandibular 406 (10%). cheek teeth and between 406-407, 409-410. CB lesion between 307-308, 308-309, 309-310, 310-311 and 406-407.
H-07	18	8 mm. PP palatally between 108-109 (Figure 19)	Large area of AL around 109 and continued to 111 (Figure 15). WS caudo-apically of 110 and 209. DI with CB lesion 34.3%) with large DI with deep PP present with most mandibular between 108 (20.1%) and 109 cheek teeth. FL lesion at 306. (14.5) (Figure 18).
H-09	18	5 mm. PP palatally between 108-109, 109-110 and 10 mm. at 208-209. DI present between 307-308 and 409-410.	FL lesion at 106 (Figure 16a) and 406 (Figure 16b). WS and AL apically of 109. Large IS (Figure 17a). Buccal AL at rostral aspect of with large DI between 208-209 (35.93%) (Figure 17a).

Abbreviation: DI = diastema; FL = inter radicular bone loss/furcation lesion; WS = widen periodontal ligament space; AL = alveolar bone loss; CB = reduced crestal bone height; PP = periodontal pocket.

apically extended to 210. DI and CB present between 306-307, 307-308, 406-407, 407-408 and 408-409. FL lesion also seen at 406.

H-10	20	5 mm. PP present	<p>Large WS and AL around palatally between 109-110, 208-209, 209-210, 210-211 and lingually between 306-307, 310-311, 407-408, 410-411.</p> <p>Large WS and AL around apical area of 109 and 209. WS lesion caudo-apically of 207. DI found between 306-307, 308-309, 406-407, 407-408 and 408-409. CB lesion between 308-309.</p>	<p>Buccal AL with DI and FL at 209 (100%) (Figure 17b). Visible FL and AL at 109 (141%).</p>
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Abbreviation: DI = diastema; FL = inter radicular bone loss/furcation lesion; WS = widen periodontal ligament space; AL = alveolar bone loss; CB = reduced crestal bone height; PP = periodontal pocket.

## Discussion

Equine PD differs from other animal species and humans due to the extended eruption process and for the ability of reattachment of the periodontal ligament. This hypsodont type of dentition with almost continuous eruption creates a scenario where the horse always has a mild gingivitis (Warhonowicz et al. 2006), which is prone to develop to the more severe chronic stage of inflammation or periodontitis. This may result in minor unspecified behavioural changes in eating, which may go unnoticed, as horses are a prey animal that do not show any signs unless severely affected (Saslow 2002). EPD diagnosis may be missed without an adequate oral examination and the use of proper equipment, such as a dental mirror and light. However, visual evaluation alone may not be enough to justify further clinical planning such as extraction of a diseased tooth or to evaluate disease progression. The most commonly used technique for confirming a diagnosis of periodontal disease in veterinary practice is radiographic imaging.

Digital radiography (DR) has been successfully introduced to small animal dentistry because it offers immediate image availability and has become a routine procedure for evaluation of severity of periodontal disease. However, sensor size availability is very restricted for equine dental use as it is either too small or too large for intraoral techniques (Intraoral sensor size 2 or full body size 25x30 centimetres). This limitation of the DR system has led equine dental veterinarians to seek other options for intraoral radiography, such as modified wet film or CR systems.

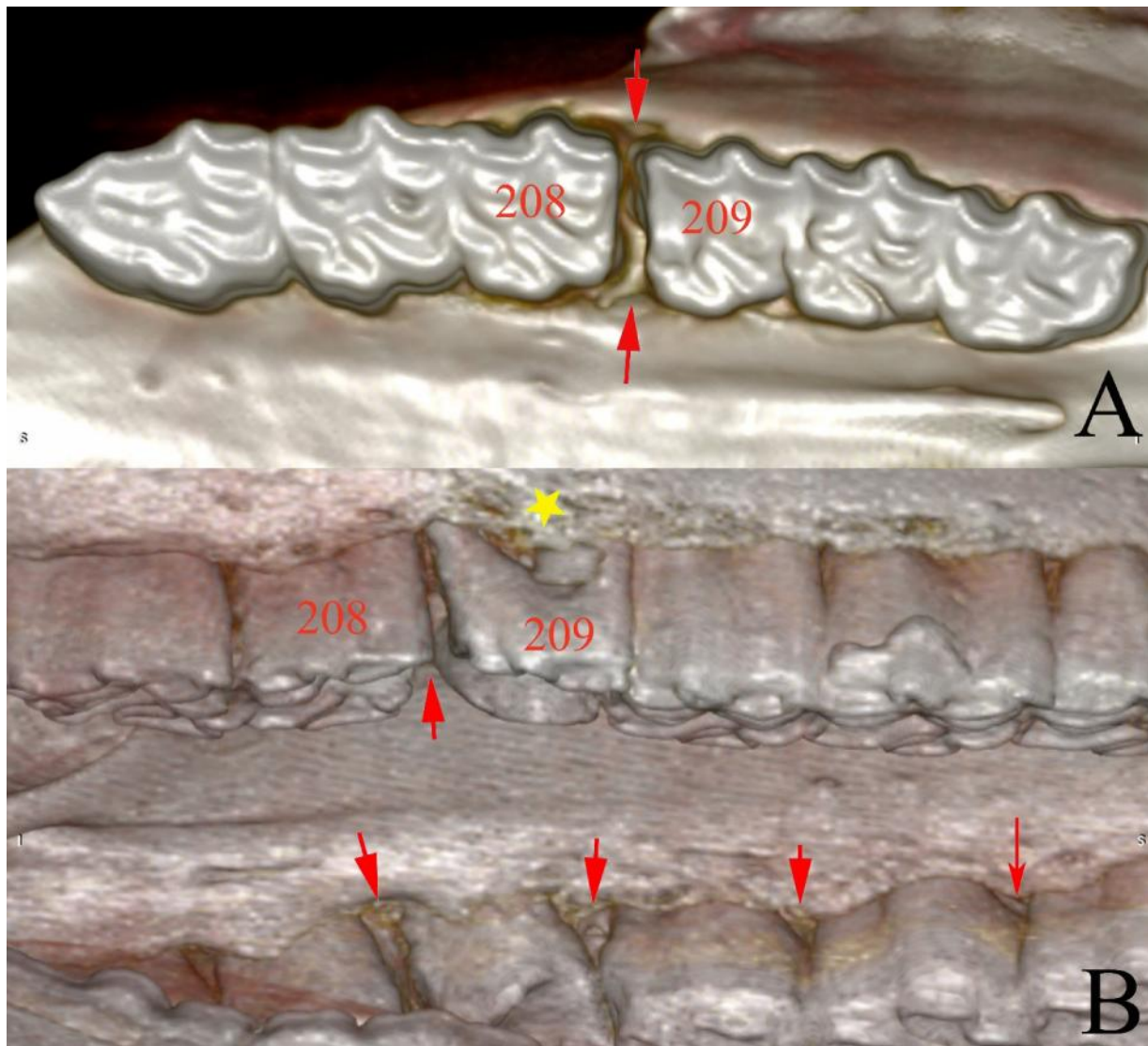
IO-CR for equine dentistry has been reviewed and discussed many times in the past decade (O'Brien 1996; Klugh 2005f; Uhlhorn et al. 2008; Baratt 2013). Some studies on the use of custom sized wet preparation X-ray film show the lack of diagnostic quality and highlight the chemical risks involved during development processes while the use of modified CR plates (cut to preferred size) has been used with good results (O'Brien 1996; Klugh 2005f; Baratt 2013). Recently, an IO-CR was developed specifically for use in equine dental radiography (CR35 VETwin, Im3 Pty Ltd. New South Wales). The benefit of this CR35 is that it comes with 3 different plate sizes (7x18 cm, 7x21 cm. and 9x21 cm.), with a slim design cassette and single use cover sheaths. A PVC handle with an indicator on the shaft helps the user when adjusting the plate to match the correct bisecting angle. In addition, handling of horses

for placement of IO-CR is always challenging, and the combination of heavy sedation, stocks and correct head support help overcome this problem without the need for general anaesthesia. The chemical free technique of IO-CR processing prevents the occupational health hazard of chemicals to veterinary professionals or technicians and reduces costs associated with building a developing room.

CT with 3D reconstruction may be more sensitive for accurate diagnostic information but the standard approach of reviewing CT images is to observe their cross sectional imaging with multiple slides of 2D images. This standard approach provides better frame by frame visualisation than 3D imaging of internal core structures. However, in this study 3D imaging produced the best visible image of periodontal lesions, which can be used for treatment planning and has been used by a German research team for an equine sinonasal study (Brinkschulte et al. 2014). The study of sinonasal communication in the horse with 3D imaging techniques has taken veterinary anatomy studies to a new level. Nonetheless, CT may be limited in usefulness due to its high cost and lack of availability. Such CT would require general anaesthesia unless a standing CT is available.

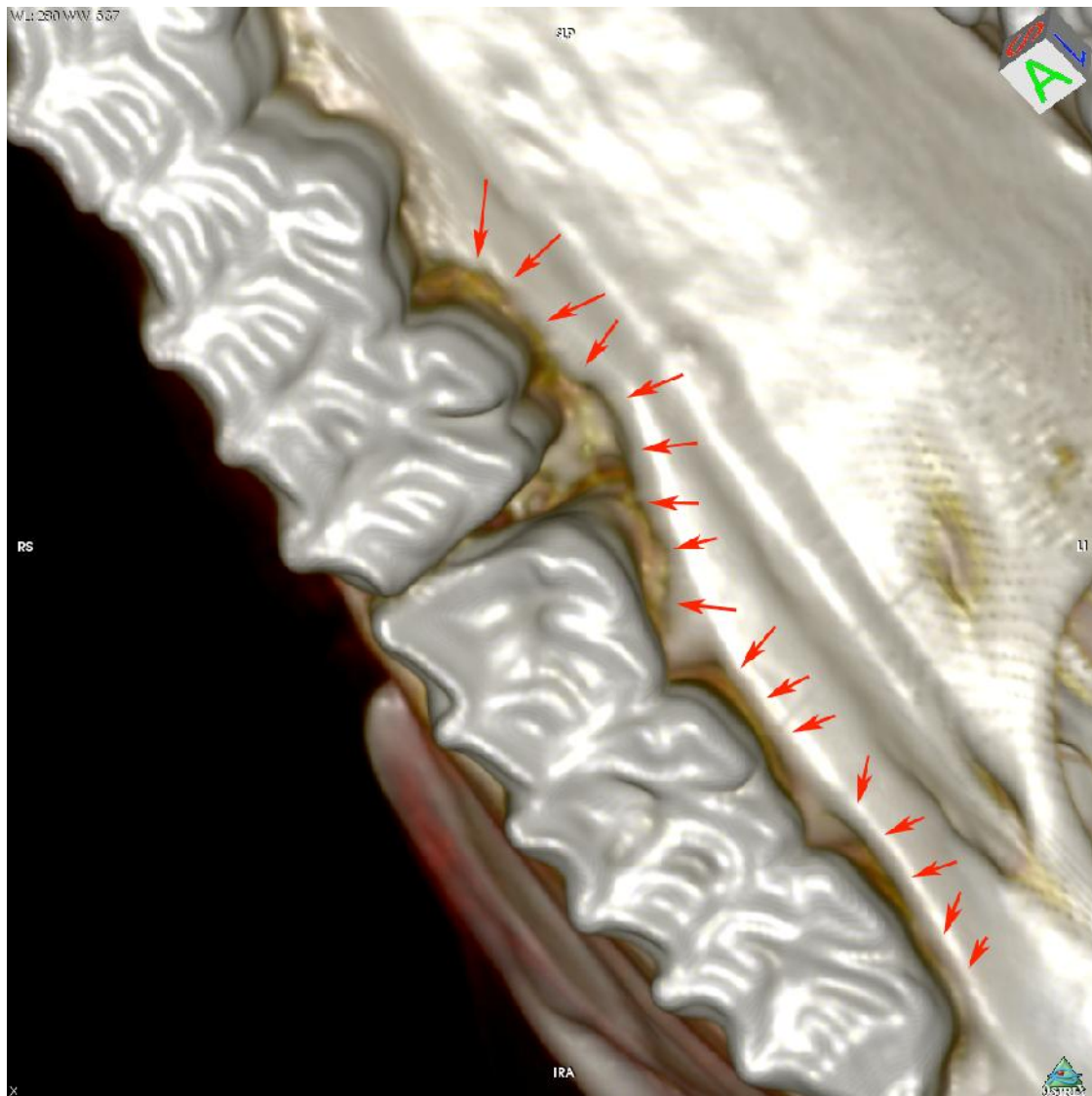
### **Furcation involvement**

One important factor for an accurate diagnosis of periodontal disease is to detect inter-radicular bone loss (furcation lesion). It is almost impossible for standard extraoral radiographic techniques to detect this lesion due to the superimposition of anatomical structures. In this study, IO-CR with good positioning showed that good diagnostic quality images can be achieved especially in upper and lower second premolar (06's) and demonstrated in the results and shown in Figure 16. On the other hand, 3D imaging also illustrates detailed bone loss and furcation lesions of severe periodontal disease, again demonstrated in this study in Figure 17b and Figure 18. This provides a reliable basis for treatment decisions as this has been used and described in human dentistry (Walter et al. 2009). The detection of this lesion in very early stages is another benefit for diagnosis and treatment planning related to unknown bit resistance problems.



**Figure 17** 3D images of periodontal lesions from horse head samples. (a) large diastema between 208 and 209 with deep periodontal pocket and alveolar bone loss around the palatal and buccal aspects of 208 (18 year old horse, H-09); (b) furcation lesion of 209 in 20 year old horse (H-10). Number, red arrows and yellow star indicate tooth nomenclature, pocket margin and furcation lesion, respectively.





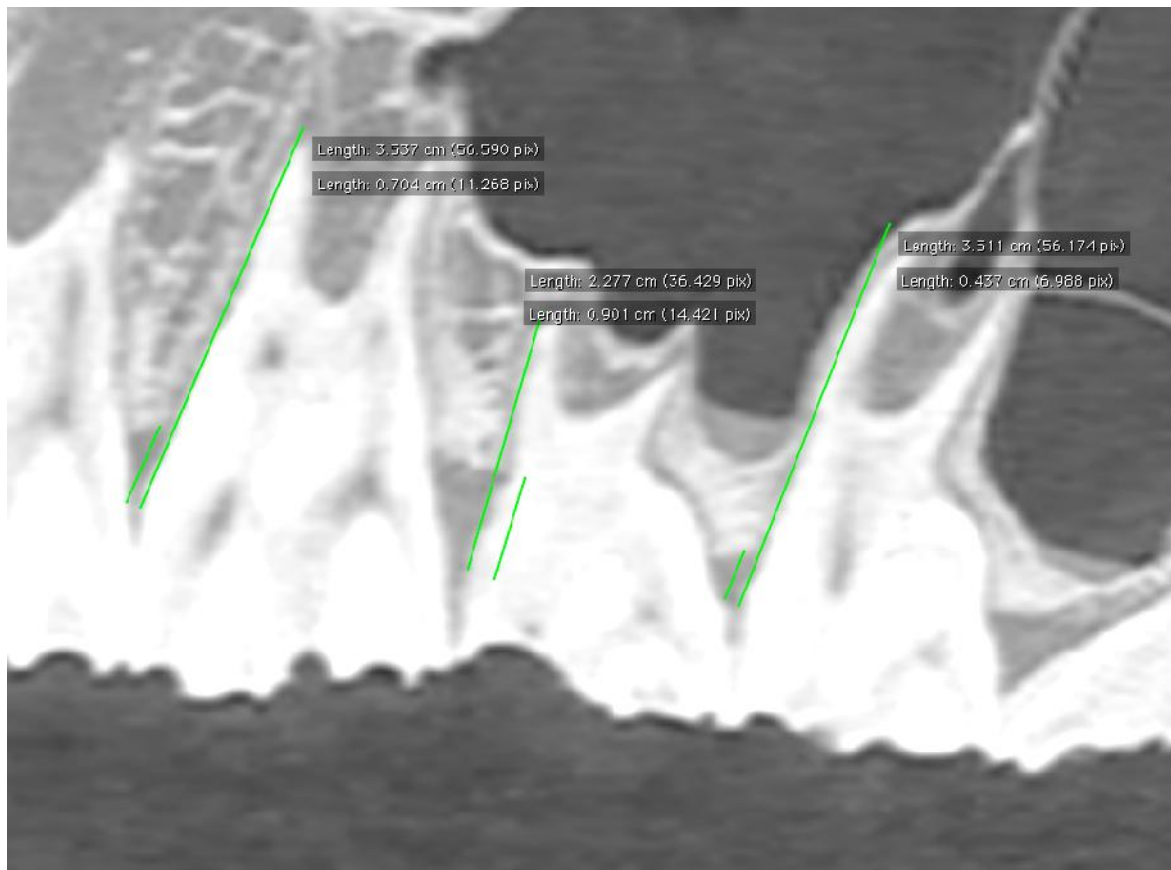
**Figure 18** 3D imaging of a large diastema between 108 and 109 with alveolar bone loss extending from the palato-rostral aspect of 108 to the palato-distal aspect of 111 (18 years old horse, H-07). Number and red arrows indicate tooth nomenclature and alveolar bone loss margin, respectively.



**Figure 19** An 8 mm. periodontal pocket of 109 presented in an 18 year old horse (H-07). Number and arrows indicate tooth nomenclature and pocket margin, respectively.

### **Periodontal ligament space**

The earliest sign of EPD that can be detected on radiographs is the increased and roughened radiolucent area of the lamina dura. In terms of the ability to produce images of periodontal ligament space, IO-CR with multi views of periapical radiographs (Figure 15) demonstrated anatomical destruction of the periodontium with far greater detail than visual examination alone. However, 3D imaging has limited visibility of internal structures of the periodontium, but this can be overcome by dissection of 3D images with more complex programing. The visibility of the periodontal ligament space using 3D imaging may not be the best solution in terms of diagnostic purpose.



**Figure 20** Attachment loss measurements of the 200' arcade (H-04).

### **Alveolar bone defects**

In a study comparing diagnostic techniques for human periodontal defects, 3D imaging was shown to have high accuracy in the detection of alveolar bone defects, whereas intraoral radiography lacked accurate measurement of the defects (Mengel et al. 2005). In a similar study comparing intraoral radiography and cone beam computed tomography, no difference was found between technologies in their ability to measure the defects (Walter et al. 2009). In this study, both IO-CR (Figure 15) and 3D imaging (Figure 17 and Figure 18) show very clear images of alveolar bone defects from different angles. However, there are limitations in 3D imaging for detecting apical lesions, which could not be seen from normal reconstructed images and will need extra work for special views.

## **Diastema**

DI is the presence of a visible increase in the interdental space where the cheek teeth are normally tightly abutted due to their eruption and angulation. IO-CR has illustrated the early signs of DI (Figure 16b, yellow star). These early stages of DI formation may not be found by oral examination; this may be another benefit of using IO-CR in routine dental examinations. 3D imaging of DI as shown in Figure 19a reveals more periodontal lesions than the DI itself. This is another benefit in terms evaluating disease progression during treatment trials.

## **Measurement of clinical periodontal attachment level**

The key element for diagnosis and follow up of periodontal disease is the measurement of periodontal attachment. In human and small animal dentistry, periodontal probing depth is the gold standard. However, this measurement cannot be applied to the horse due to its different dental anatomy (reserve crown and root length vary with age, and no clear point of cemento-enamel junction). By using CT images with a DICOM image viewer that has a function for measurement, CAL in horses can be measured with acceptable accuracy depending on slice thickness at the time of the scan. Klugh (2005a) described the guideline for assessment of percentage of attachment loss adapted from intraoral radiography of brachydont teeth. There are some limitations with intraoral radiography for estimating where the periodontal lesion ends as superimposition of the tooth itself is common. This limitation can be overcome by using CT images where superimposition has no effect on the image quality.

By comparison of the three methods used in this study oral examination is the most simple and only requiring minimal tools. This method can be considered as initial method for detection of periodontal abnormalities. While a secondary diagnosis technique the IO-CR can be used which will provide a more in depth detail of each specific lesion. However, IO-CR cannot be used for the routine scanning of the lesion, as the technique requires specific radiographic infrastructure for each location and there is a risk of radiation from excessive use. The 3D imaging technique was shown to be significantly more sensitive in diagnosis of specific lesions and with the ability to accurately calculate attachment lost. There are limitations to be considered such as the cost per procedure, availability of a CT machine for horses and anaesthetic risk.

In conclusion, the new generation of IO-CR for horses has demonstrated potential as an effective auxiliary tool for diagnosing EPD of cheek teeth. With adequate knowledge of beam angle and plate position techniques, this can become a routine procedure for equine dentistry. However, IO-CR will never replace the need for extra oral radiography nor computed tomography, but the use of these techniques will definitely increase sensitivity for detecting and evaluating equine dental pathology.

## **Chapter 6:**

**The effect of a chlorhexidine gluconate mouth rinse on equine oral microbiota determined by next-generation sequencing.**

## Introduction

EPD is one of the most common dental diseases in domesticated horses with almost one fourth of domesticated horses suffering from this condition (Simhofer et al. 2008; Anthony et al. 2010). Currently in equine dental practice, this condition is best controlled by periodic dental care. This involves mechanical removal of plaque accumulation with brushing and scaling, and the use of an antiseptic oral rinse or tooth paste. Mechanical plaque removal, under sedation, includes a complete oral examination, reducing sharp enamel points and flushing of the oral cavity with water, with or without antiseptic. Control of EPD, however, is difficult to achieve in a single visit and requires on-going daily or weekly antiseptic treatments. The most common chemotherapeutic agent for this purpose is chlorhexidine gluconate (CHX). CHX is a biguanide broad spectrum antimicrobial developed by a British company (Imperial Chemical Industries; Pharmaceuticals division, Manchester, United Kingdom) during the mid-1900s. It has a significant history of safe and effective use for oral health control in humans and animals (Davies et al. 1954; Rindom Schiøtt et al. 1970; Schemehorn et al. 1982; Briner et al. 1986; Sreenivasan & Gittins 2004; Tomas et al. 2008). CHX was used in a canine model to investigate teeth staining (Schemehorn et al. 1982). While CHX has been used in veterinary dentistry and is available as a commercial oral rinse for horses (Hexarinse®, Virbac (Australia) Pty Ltd, Milperra New South Wales, Australia), the effect of CHX on the treatment of EPD and its effects on equine periodontal bacteria have never been studied.

The study of microbiota using culture-dependent methods under represents the diversity of bacterial species recovered due to stringent requirements necessary to cultivate some of these oral bacteria. Next generation sequencing (NGS) and metagenomic analysis has been shown to identify a broader and more in-depth level of genus and species detail in complex microbial ecosystems (Simon & Daniel 2011).

Therefore, the objective of this study was to determine the effect of Chlorhexidine gluconate (1.4% w/v) on the equine microbial community using NGS (MiSeq System, Illumina). Changes in the equine oral microbiome was determined after three months of continued oral rinse usage to understand the potential benefit of CHX in the treatment of equine periodontal disease.

## **Materials and methods**

### **Animal and sample collection**

Twelve horses of mixed breed and gender, and with no history of dental treatment in the previous six months, were selected for the study. Horses from four to 23 years of age each having at least some degree of EPD, based on the descriptions of Dixon and Dacre (2005). Horses were obtained from the School of Veterinary Science University of Queensland's teaching herd. This study had animal ethics approval for the use of horses as experimental animals from the Queensland University animal ethics committee (No. SVS/161/12). A complete dental examination was performed on each horse at the start of the study. Horses were divided into three groups (four in each group): control group (Ct), which was subjected to only a dental examination without any physical or chemical treatments; a water treatment group (Wt); and CHX treatment (CXT) group. The treatment groups were subjected to routine dental floating followed by an oral rinse every two days for three months with 60 milliliters of water or CHX 1.4% w/v (Hexarinse®, Virbac Pty Ltd), respectively (see video clip in Appendix for Chapter 6; Hexarinse, make it easy for horse dental care.wmv).

Post treatment plaque samples were collected 24 hours after the last rinse using No: 90 sterilized paper points placed in the gingival sulcus for 30 seconds with the aid of 47.5 cm sterilized equine periodontal forceps. The paper points were then transferred into sterilized cryovials containing 1 ml of reduced transport medium and frozen immediately in liquid nitrogen for transport. All samples then transfer for storage at -80°C freezer until further processing. The transport medium was prepared using WCB (Oxoid Australia Ltd. West Hedelberg, Victoria, Australia) with added glycerol (10%). The medium was autoclaved, cooled to room temperature, and then 1 mL aliquots were transferred into cryovials. The vials were placed in an anaerobic atmosphere of 86% N<sub>2</sub>, 10% CO<sub>2</sub>, and 4% H<sub>2</sub> then left overnight to reduce the medium.



## **DNA extraction, PCR amplification of bacterial 16S rRNA gene, library preparation and next generation sequencing**

NGS was performed as described in Chapter 4. Briefly, bacterial DNA was extracted from each plaque sample using the Powersoil® DNA Isolation Kit (Mobio Laboratory, Carlsbad, CA) following the manufacturer's instructions, then verified and quantified by Qubit® Fluorometer using the dsDNA BR Assay kit (Life Technology). The V6 to V8 16S rRNA gene was targeted using primers 803F and 1392R, then the 16S rRNA library and data processed following the Illumina® workflow. The sequence data were grouped into operational taxonomic units (OTUs) 97% similarity threshold, and OTUs with less than 0.05% relative abundance were removed. Representative OTU sequences are annotated by BLASTing against the Greengenes reference database(version2013/05;[http://greengenes.lbl.gov/cgi-bin/nph-ompare\\_choices.cgi](http://greengenes.lbl.gov/cgi-bin/nph-ompare_choices.cgi)).

### **Statistical analysis of NGS**

Shannon and Simpson diversity index of all groups was calculated with R software and t-tests ( $p$ -values at 0.05) for statistical significance were performed using SPSS version 20. The construction of “heatmaps” and determination of relationships in the microbial community data were performed using PERMANOVA. OTUs with a 97% similarity threshold and presence of more than 1% relative abundance was determined using R package gplots (Dixon 2003; Warnes et al. 2014).

## **Results**

### **Sequencing data**

Sequencing generated a total of 1,899,606 reads for all 12 samples, with a mean of 158,300 sequences per sample (Table 7).

After removing sequence errors and cleaning using R Studio, the dataset decreased by approximately 49.5% to a total of 958,679 sequences and an average of 79,890 sequences per sample, (65,141– 112,666; standard deviation  $\pm 16,330$ ) was observed.

## OTU-based analysis

Across all samples a total of 198 OTUs were clustered. Only six percent of the OTUs could not be identified at the genus level and were classified at the next highest taxonomic level. Only 47 OTUs out of the total OTU presented with a relative abundance of at least 1% belonged to the domain Bacteria and represented 11 different phyla, including Bacteroidetes (35.4%), Proteobacteria (25%), Firmicutes (19.8%), Fusobacteria (15%), Actinobacteria (1.5%), SR1 (1%), TM7 (0.2%), Synergistetes (0.1%), Tenericutes (0.1%) and Cyanobacteria (0.005%). The relative distributions of 47 OTUs is illustrated as Heatmaps (Figure 21). Statistical and diversity index analysis

Shannon and Simpson diversity indexes and OTUs numbers are also presented in Table 7 showing no statistically significance among groups. Meta data were visualized using PCA plot describing dissimilarity (Figure 22) and showed that all of 47 OTUs were clustered in the centre of the plot without any relation to any specific group or sample (control, Wt or CXT). It is also confirmed that there was no statistical difference on distribution of sequences ( $P = 0.913$ ).

By comparing the average relative abundancy of sequences at phylum level to our previous study in Chapter Four, results in this study showed that bacteria from phylum Bacteroidetes from CXT was lower compared to those periodontal affected group (PD) but not statistically different (Figure 23).

## Discussion

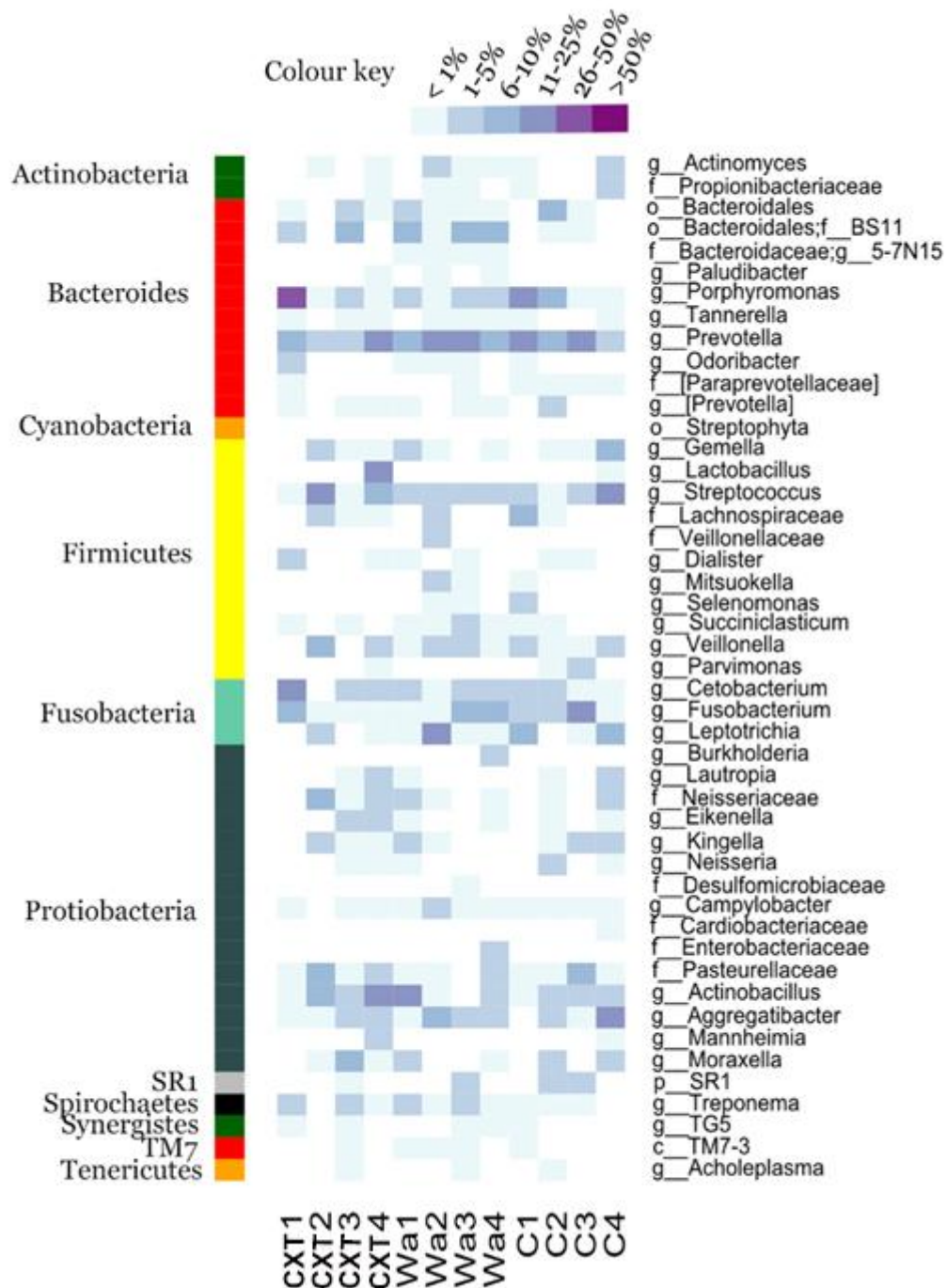
CHX has been used as a therapeutic agent for skin and oral disinfection in veterinary science for more than 30 years (Briner et al. 1980; Foulkes et al. 1973; Rindom et al. 1970). It has been clinically proven in dentistry to reduce the risk of gingivitis and periodontitis, with the reduction in the loss of attachment and alveolar bone (Briner et al. 1980; Tepe et al. 1983). Nonetheless no reports in the literature demonstrated the effect of CHX on EPD. This study is also the first *in-vivo* study to determine the effect of CHX on equine oral microbiota using NGS. The reduction of organisms representing the phylum Bacteroidetes in the CXT group (33%) was less

when compared with the periodontal disease horse group (43%; Chapter 4) following the treatment period. It has been reported that CHX can inhibit haemagglutination and alter the adherence of *P. gingivalis* and thereby reduce these species in human PD (Grenier 1996). Because species level detection was unavailable in the present study, it is unclear whether or not a similar aetiology is occurring. However, future analysis may show a reduction in equine specific species within the genus *Porphyromonas* that maybe associated with EPD.

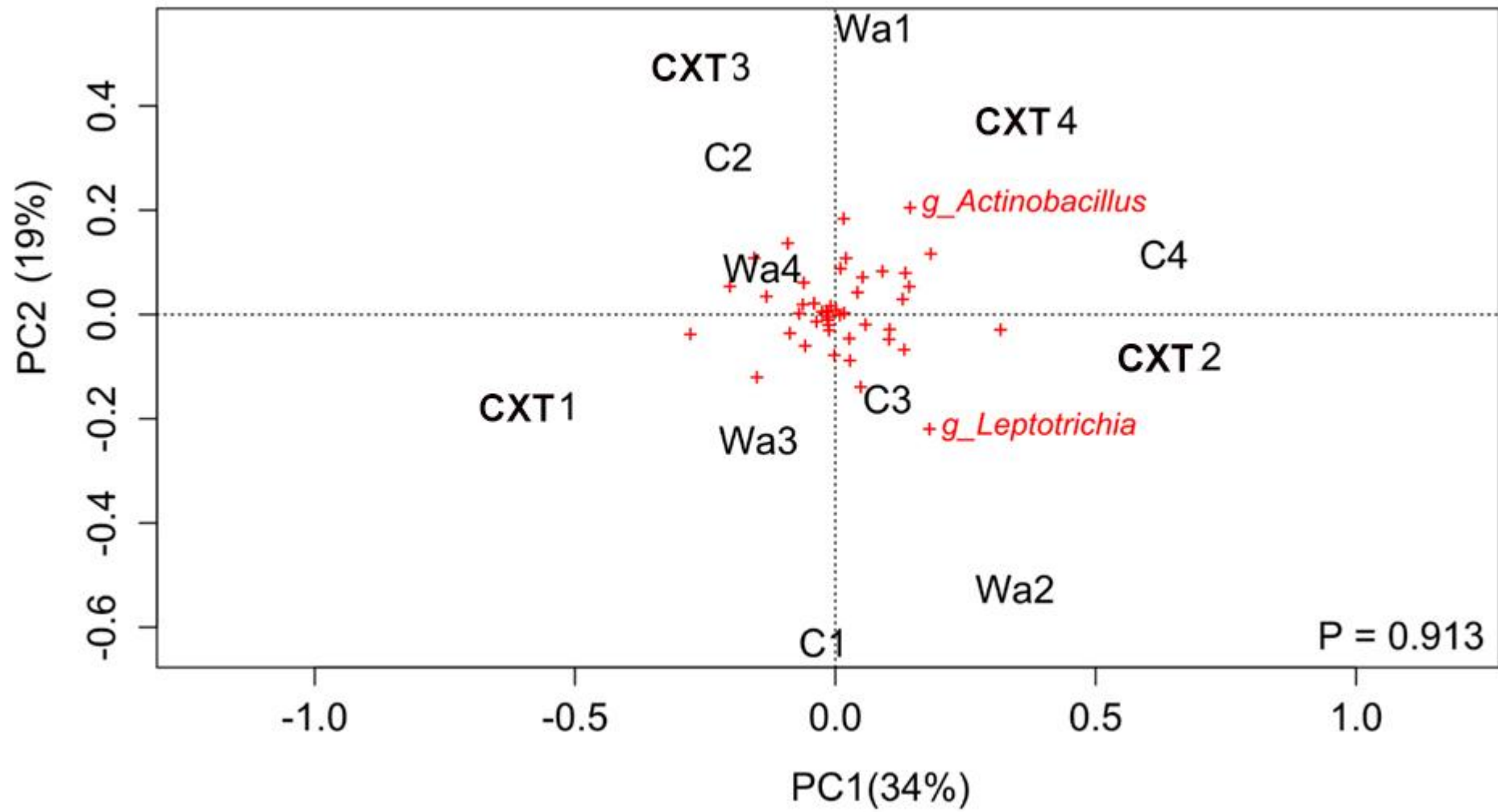
The average relative abundance along with bacterial diversity indexes (from more diverse to less diverse) for the equine are similar to previous studies in canine and human, which had longer treatment periods (six months to seven years), where significant results in reducing plaque accumulation, gingivitis index and sulcus depth were observed (Briner et al. 1980; Briner et al. 1986). However, Briner et al. (1986) showed that in the first three months there was no difference in bacterial communities between CHX and placebo trial groups in human. Then after three months, a reduction in the bacterial community of the CHX treatment group occurred, which ranged from 55% to 97%. The minor changes observed in our study may have resulted from the inadequate dosage, lower concentration of CHX or a shorter period in the treatment trial. There are consequences of using long term oral antiseptic agents to prevent and treat EPD, such as whether or not the agent could produce a shift in the gastrointestinal microbial ecosystem, which may lead to colic or laminitis. In addition, microbial resistance to CHX could also be possible as there are reports of methicillin-resistant *Staphylococcus aureus* in human patients using CHX skin soap (Brooks et al. 2002; Fritz et al. 2013).

Another parameter that is useful for evaluating the effectiveness of treatment for EPD is periodontal ligament reattachment gain. Some periodontal topical antibiotics, such as doxycycline, have been proven to increase gingival attachment between 40-50% in pockets with more than 4 mm depth within 12 weeks of treatment (Polson et al. 1996; Zetner & Rothmueller 2002). In the present study the effectiveness of CHX for stimulating periodontal attachment was not evaluated due to the limitation of using an equine periodontal probe. True periodontal attachment must be determined by intraoral radiographic images (as discussed in Chapter five or Five (need consistency). Determination of periodontal attachment in future research will allow for precise evaluation of the effectiveness of chemical therapeutic agents on PD.

In conclusion, the use of 1.4% w/v CHX oral Hexarine® showed a drift to a less diverse bacterial community, which was not statistically significant when compared to control groups after a three month treatment trial. In consequence, the duration, dosage and interval between applications seem to be important factors, although the use of long term, high oral doses of CHX may have negative consequences due to induced changes in intestinal bacteria, and this needs further investigation.



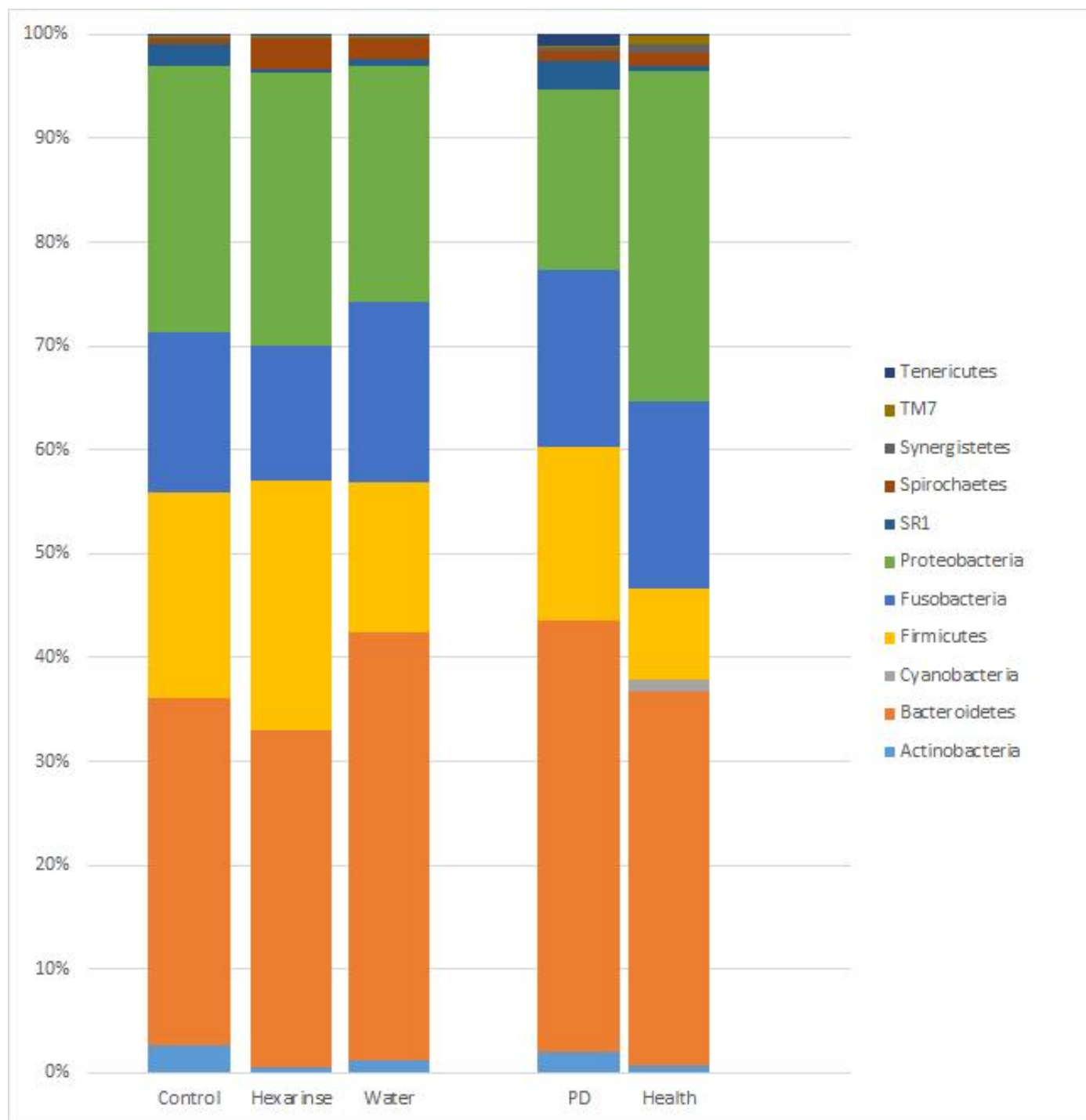
**Figure 21** Genus-level relative abundance of bacterial isolated from 12 plaque samples collected from horses. Taxa shown are at genus-level (g\_) or at next lowest taxonomic classification. C = control group without any oral rinse, CXT = oral rinse with Chlorhexidine gluconate, Wa = oral rinse with water only.



**Figure 22** Principle component analysis of horse plaque samples with next generation sequencing. P value calculated using PERMANOVA.

**Table 7** Diversity statistics of bacterial communities isolated from 12 horses treated with Hexarinse (CXT), Water (Wt) and a Control group (C) as determined by next generation sequencing.

	<b>CXT 1</b>	<b>CXT 2</b>	<b>CXT 3</b>	<b>CXT 4</b>	<b>Wt1</b>	<b>Wt2</b>	<b>Wt3</b>	<b>Wt4</b>	<b>C1</b>	<b>C2</b>	<b>C3</b>	<b>C4</b>
<b>Total sequences</b>	146475	124695	180125	231736	128031	164284	133138	208761	120519	139152	143503	179187
<b>No. OTUs</b>	128	130	143	147	149	149	133	69	119	136	154	149
<b>Simpson</b>	0.78850	0.82870	0.92011	0.86410	0.89207	0.87034	0.85370	0.89319	0.86263	0.91113	0.79093	0.88017
<b>Shannon</b>	1.97825	2.16561	2.82550	2.48522	2.66627	2.52743	2.54314	2.69912	2.34251	2.79098	2.05652	2.53855



**Figure 23** Phylum level comparison of horse oral microbial communities after three months of different oral rinse protocols compared to a previous study (see Chapter 4). PD = periodontitis group.



## **Chapter 7:**

### **General discussion and future consideration**

## General discussion

The aims of this thesis were fourfold: (1) to survey horses from South-East Queensland region of Australia to determine the prevalence of dental disorders, (2) to determine microbiota of the subgingival plaque sampled from horses with healthy gingivae and horses with periodontitis, using culture dependent and culture independent methods; (3) to diagnose EPD using intraoral radiography and 3D imaging techniques; and (4) to investigate a treatment regime for EPD in horses using a CHX mouth rinse and then determine the effect of CHX on the oral bacteria using next generation sequencing.

The results of this study showed that 93.8 percent of Australian domesticated horses had one or more type of dental abnormality, with the most common being sharp enamel points. Other dental abnormalities, such as the presence of periodontal pockets, an indication of EPD, diastema and missing tooth, were present in the older horses. Differences in the incidence of sharp enamel points have been reported in the literature, but these may be influenced by differences in definition or examination techniques (suitable equipment, sedation protocol and the use of palpation and visual examination) (Simhofer et al. 2008; Anthony et al. 2010; O'Neill et al. 2010; Pathomsakulwon et al. 2011).

In this study of oral microbiota of horses, using culture dependent methods 116 bacterial species from 16 genera were isolated and identified using 16S rRNA sequencing. With NGS a total of 592,019 bacterial sequences were recovered, with a total of 46 OTUs, but a limitation of the Illumina software precluded species level identification. Even so, the equine periodontal microbiota was shown to have a unique diversity compared to other animals, such as dogs (Riggio et al. 2011; Sturgeon et al. 2013) and cats (Sturgeon et al. 2014). This may due to differences in diet and dentition, as horse teeth have a long eruption time. The equine oral microbial community was similar in horses with healthy gingiva and periodontal disease, but with disease a narrower range of diversity was observed.

The recovery rate of bacteria and bacterial identification differed between culture dependent and independent methods. The culture dependent methods showed less diversity but more precise bacterial identification to species level, which is required to determine which bacterial pathogen(s) are associated with disease. On the other

hand, NGS identified a broader range of bacterial diversity, but bacteria were unable to be identified to species level with the current software used in this study. With improvements in techniques and instrumentation, species-level bacterial microbiota will be identifiable in the future. Culture-dependent methods have limitations with growth and media requirements, but in combination with NGS, bacteria can be grown and then genomes sequenced to obtain greater clarity, as demonstrated in a recent canine oral microbiome study (Coil et al. 2015).

The clinical diagnosis of equine periodontal disease is based on an oral examination using traditional visualisation with tools, such as a dental mirror, pick, and probes. One criteria of periodontal disease is the loss of attachment, and in EPD this cannot be determined by simple measurement of the depth of the periodontal pocket as the hypsodont root and reserve crown vary with age. For this reason, the visibility of each length of root and reserve crown along with the depth of periodontal pocket must be calculated as a percentage of attachment loss. Digital radiographic imaging of the periodontium gives a precise percentage loss as it provides a clear view of the tooth and surrounding structures. The gold standard for diagnostic imaging in equine dentistry is computed tomography (CT) but availability and cost severely limit CT's use. Wireless digital radiography (DR) provides the next best alternative method, producing a quality images within a few seconds. However, adoption of this technology to equine dentistry has limitations due to the size of the sensor, which restricts its use to the extra-oral technique, where image distortion is unavoidable. An alternative radiographic method is intraoral computed radiography (IO-CR), where a small radiographic film plate with high image resolution is modified to fit inside the oral cavity. IO-CR has been previously discussed and reviewed (O'Brien 1996; Klugh 2005f; Uhlhorn et al. 2008; Baratt 2013) but in these studies, the plate was modified or cut out from full body film. The recent introduction of a commercially available equine IO-CR (CR35 VETwin, Im3 Pty Ltd. New South Wales) provides a choice of three plate sizes with a protected cassette. In this study, IO-CR produced a unique view of dental lesions that were not evident using extraoral radiography. IO-CR allowed determination of furcation involvement and diastema of the lower cheek teeth arcade, the apical region of upper third molar in young horses, and the ability to measure accurately the percentage of periodontal attachment loss, which is the key element for diagnosis and follow up of periodontal disease. IO-CR therefore allows the equine

dental veterinarian to monitor disease progression and the patient's response to treatment through comparison of the pre and post treatment radiographs.

Maintaining good oral health and treating equine periodontal disease can be achieved in two ways: 1) routine mechanical control, including regularly reducing sharp enamel points and correcting malocclusion, 2) chemical control, such as an oral antiseptic rinse. The most common chemotherapeutic agent for the oral cavity is chlorhexidine gluconate, which has been proven to be effective in controlling oral microbial populations in both humans and animals (Davies et al. 1954; Rindom Schiøtt et al. 1970; Schemehorn et al. 1982; Briner et al. 1986; Sreenivasan & Gittins 2004; Tomas et al. 2008). No studies on the effectiveness of CHX in horses were reported before this study. Next generation sequencing results from this study demonstrated that CHX treatment reduced the relative abundance of bacteria from phylum Bacteroides within first three months but the reduction was not statistically significant. This may be due to the short trial duration (only three months), inadequate dosage, or incorrect interval between applications limiting the outcome of the results. It has yet to be determined if long term oral use of CHX has collateral effects on the survival of intestinal microflora, which may lead to other problems such as colic or laminitis.

### **Future considerations**

This thesis constitutes a comprehensive surveillance study on equine dental abnormalities, equine oral bacteriology, intraoral radiography and the effectiveness of chlorhexidine gluconate as a horse oral rinse. As discussed, the results from this body of work have suggested future research directions to investigate the epidemiology, radiographic techniques, prevention and treatment of EPD. Successful completion of the aims of this thesis could not definitively determine which bacterial species are involved in the aetiology of equine periodontal disease. However, it has implicated a group of microorganisms that could be associated with disease. Another study, which is currently being carried out, is a longitudinal study of the microbiota of oral plaque in foals from birth to 12 months of age to determine the microbiota of the developing plaque, as well as to compare the microbiota of the plaque from mares in the same herd. The results of this thesis have provided a foundation for future work to determine the periodopathogenic bacteria associated with and perhaps the aetiological agents of equine periodontal disease.

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## Appendices

**Appendices to chapter 3**

An abattoir survey of equine dental abnormalities in Queensland, Australia.

This appendix is presented in the form of a scientific paper, unedited from the original uncorrected proof version:



## An abattoir survey of equine dental abnormalities in Queensland, Australia

T Chinkangsadam,\* GJ Wilson, RM Greer, CC Pollitt and PS Bird

**Objective** A cadaver study to estimate the prevalence of dental disorders in horses presented at an abattoir in Queensland, Australia.

**Methods** Cadaver heads at a Queensland abattoir were examined for the presence of dental abnormalities and categorised into age groups. The prevalence of abnormalities was analysed by binomial observation of observed proportion, Pearson's Chi-square test or Fisher's exact correlation test. Strength of association was evaluated using Cramer's V test.

**Results** Heads from horses ( $n = 400$ ) estimated to be between 1 and 30 years of age were placed into four age groups. The most common abnormalities were sharp enamel points (55.3%) and hooks (43%). The highest frequency of dental diseases and abnormalities were in horses 11–15 years old (97.5%).

**Conclusions** Common abnormalities were found in all groups and the prevalence increased with age. This study suggests that all horses should have regular complete dental examinations to detect and treat dental disorders in order to limit more severe dental pathologies later in life.

**Keywords** abattoir survey; dental abnormalities; equine dentistry; horses; Queensland

**Abbreviations** CI, confidence interval; ETR, exaggerated transverse ridges; OR, odds ratio; PD, periodontal disease; SEP, sharp enamel points

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Dental diseases are common oral disorders in horses.<sup>1</sup> Surveys have reported that 10% of cases presented to equine practices in England were dental related<sup>2</sup> and dental cases were in the top five medical problems in adult horses in the USA.<sup>3</sup> Of 400 horses referred to an equine clinic in Scotland because of dental disorders, 87% of cases were diagnosed with primary disorders of the cheek teeth, including 44 cases (12.6%) of gross abnormalities of wear, of which 5 (11.4%) of the horses surprisingly exhibited no clinical signs. Other types of disorders such as traumatic damage, idiopathic fractures and dental tumours always presented with clinical signs.<sup>4</sup> Other dental-related surveys on both live horses and post-mortem specimens have shown clinically significant findings on dental abnormalities in horses.<sup>5–9</sup> For example, a survey of 556 horse cadavers examined in a Canadian abattoir showed that 70% had at

least one type of dental abnormality of their cheek teeth. The most common dental disorder was sharp enamel points (SEP) (47.7%) and 36.2% of horses with SEP had buccal abrasions.<sup>7</sup>

Australia has an estimated 1.2 million horses<sup>9</sup> and to our knowledge a targeted detailed equine dental survey has not been conducted of an Australian horse population. A general health questionnaire administered to owners of horses aged  $\geq 15$  years in Queensland, Australia<sup>10</sup> showed that 9% of the horses were reported by their owners to have clinical signs of dental disease, which can contribute to weight loss, head and mouth pain, behavioural abnormalities and poor performance.

In Queensland, aged, unwanted or retired horses from a broad range of sources are sent to an abattoir. The aim of this study was to investigate the prevalence and association of dental disorders in this population of horses.

### Materials and methods

#### Study design and horses

The study was a cross-sectional survey of dental abnormalities in horse heads. It was conducted from November 2011 to January 2012 at an abattoir in Caboolture, Queensland, Australia, which has a catchment area of mainly south-east Queensland. It exports horse meat for human consumption. Horse breeds included Thoroughbred, Standardbred, Stock Horse and mixed/cross-breeds. Sick or severely injured horses are not accepted for slaughter. The survey was conducted fortnightly and all horses on a particular day were studied.

The study was approved by the Animal Ethics Committee of the University of Queensland (ANRFA/SVS/331/13).

#### Head and dental examination

The tongue and most of the facial and masticatory muscles, lymph nodes and salivary glands were removed during the slaughter process. The heads were examined at the abattoir premises, starting with visual examination of skull symmetry followed by flushing of the oral cavity to clear all debris to increase visibility.

Horses were grouped into four age groups (0–5 years, 6–10 years, 11–15 years and  $>15$  years) using the appearance of dentition, as described elsewhere.<sup>11,12</sup> Categories used were stage of teeth eruption for horses aged  $<5$  years old; loss of dental cups on incisors and shape of occlusal table for horses aged 6–10 years; persistence of central enamel for horses aged 11–15 years; and loss of central enamel for horses aged  $>15$  years.

All dental examinations were made visually and by palpation, and started with identification of incisor abnormalities, including bite

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alignment (categorised as normal alignment, overshot/parrot mouth and undershot/sow mouth) and presence of canine abnormalities. The cheek teeth were closely examined on all surfaces (buccal, palatal, lingual, labial and occlusal) and the presence of the first premolar (wolf teeth) noted. More descriptions of dental abnormalities are described elsewhere.<sup>4</sup>

The gingivitis index could not be defined because of postmortem gingival discolouration and lack of gingival bleeding on probing. Only visual periodontal pockets (food and material packed into the gingival crevice) and diastemata (visible spacing between adjacent teeth) were recorded.

Other data recorded included presence of SEP (>1 mm from the level of the occlusal surface), mobile teeth, fractured teeth, abnormalities of wear and eruption such as stepped teeth, hooks, ramps and exaggerated transverse ridges (ETR).

#### Dental abnormalities recording and charting

All findings were collected using a voice recorder in the working environment, then transferred to a dental chart using a modified Triadan tooth numbering system<sup>13</sup> using the Equine Veterinarians Australia equine dental chart.

#### Statistical analysis

The percentage prevalence (number of heads with dental abnormalities ÷ number of heads examined × 100) of dental abnormalities were estimated with a 95% confidence interval (CI). Association with age group was evaluated using Chi-square test, or Fisher's exact test if the cell frequency was <5. Strength of association was evaluated using Cramer's V, where >0.5 indicates high association, 0.3–0.5 moderate association, 0.1–0.3 low association and 0–0.1 little if any association.

The average age of affected and non-affected was compared using a t-test for independent samples. SPSS (Version 20) was used for analysis.

#### Results

Because of the standard abattoir protocols, and safety concerns for visitors, the examiner was not allowed access to the holding area to examine the live horses for sex and general health. From a distance the horses appeared to be of various breeds including Standardbred, Thoroughbred, Stock Horse and mixed/cross-breeds. There were no feral horses in this survey. All horses appeared to be healthy, because sick or severely injured horses are excluded from the slaughter process.

All 400 heads were examined and included in the analyses. Dental age ranged from 1 to 30 years (median, 8 years) (Table 1).

#### Dental abnormalities

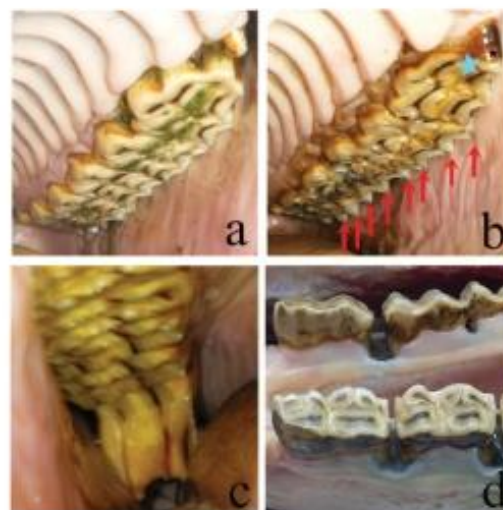
No skull asymmetry was observed. Any asymmetry of the masticatory muscles could not be evaluated because of their removal during the slaughter process.

A total of 375 heads (93.8%) were found to have one or more dental abnormality; 25 heads (6.7%) were without any dental abnormalities. Details of the frequency of dental abnormalities are shown in Table 1.

**Table 1.** Frequency of dental abnormalities observed in 400 horse skulls from south-east Queensland, Australia

Dental abnormality	n	Proportion (%)	95% CI
No abnormality observed	25	6.2	4.1–9.1
SEP	272	68	63.4–72.6
Hook	172	43	38–48
Wave mouth	102	25.5	21.3–30.0
Periodontal pocket	89	22.3	18.3–26.7
ETR	54	13.5	10.3–17.2
Diastema	52	13	9.9–16.7
Ramp	30	12.5	9.4–16.1
Retained deciduous tooth	44	11	8.1–14.5
Stepped tooth	34	8.5	6–11.7
Missing tooth	21	5.3	3.3–7.9
Fractured tooth	16	4	2.3–6.4
Mobile tooth	11	2.8	1.4–4.9
Cupped out tooth	49	12.3	9.0–15.5

CI, confidence interval; ETR, exaggerated transverse ridges; SEP, sharp enamel points



**Figure 1.** Normal and common equine dental abnormalities. (a) Normal teeth occlusion in 5-year-old horse, (b) sharp enamel point (arrows) and hook (star) in 6-year-old horse, (c) stepped tooth in 12-year-old horse and (d) diastemata/periodontal pockets with visible soft tissue and bone loss in 1-year-old pony.

#### Frequency of incisors bite alignment, canine abnormalities and presence of wolf teeth

The frequency of incisor alignment, defined as normal bite alignment, was 79.9% (95% CI, 75.5–83.6); overshot was seen in 20.0% (95% CI, 16.2–24.3) of the heads and no cases of undershot were



observed. Two fractured canines were found at positions 204 and 304; 22.75% (95% CI, 18.7–27.1) of horses had one or more wolf teeth.

#### Abnormalities of wear and overgrowth

The normal occlusal surface of a young horse is shown in Figure 1a and abnormalities of wear and overgrowth such as SEP, hook, stepped teeth and periodontal pockets are shown in Figure 1b–d.

The majority of hooks (98.4%; 95% CI, 96.5–99.4) were found in the cheek teeth. Of other abnormalities found, the commonest positions of stepped teeth were at the lower right mandibular fourth premolar (position 408; 35.1%; 95% CI, 20.2–52.5) and at the lower left mandibular fourth premolar (position 308; 29.7%; 95% CI, 15.9–47.0%). In addition, 40% (95% CI, 29.5–50.2%) of ramps were found in the lower left mandibular second premolars (position 306) and 38.8% (95% CI, 28.4–50.0%) were found at the lower right mandibular second premolar (position 406).

#### Periodontal pockets and diastema

There were 144 periodontal pockets observed in 89 heads (Table 1), mostly interproximally (between adjacent teeth); only one periodontal pocket was found on the palatal side of one upper left fourth premolar (position 208). Periodontal pockets were found most commonly in the interdental space between the fourth premolar and the first molar (41.67%; 95% CI, 33.5–50.2), followed by the molar area (32.64%; 95% CI, 25.1–40.9), the premolar area (22.2%; 95% CI, 15.7–30.0) and the incisor area (2.8%; 95% CI, 0.8–6.9) (Figure 2).

There were 107 diastemata found in 52 heads (Table 1), 40.2% (95% CI, 30.8–50.1) of which were interproximally located between incisors (Figure 3).

A total of 13 mobile teeth were found, with no specific location in the mouth noted.

#### Age and dental abnormalities

The proportion of abnormalities differed according to the age group (Table 2). Horses aged 0–5 years had a higher prevalence of retained deciduous teeth and ETR (Figure 4a) than any other age group (Table 2).

Horses aged 11–15 years had a prevalence of hooks of 59.2% (Table 2) with an odds ratio (OR) 3.7-fold higher than the reference group (0–5 years horses) (Table 3); 30.8% had wave mouth (Figure 4b); 12.5% had stepped teeth; 14.2% (95% CI, 8.5–21.7) had ramps and 6.7% (95% CI, 2.9–12.7) had fractured teeth (Figure 4c).

Horses aged >15 years had a prevalence of diastema of 40.8% with an OR of 7.7-fold higher than the reference group; 12.2% (95% CI, 4.6–24.8) had mobile teeth and 18.4% had missing teeth, with an OR 7.3-fold higher than the reference group (Table 3).

Comparison of proportions by Chi-square test for each group showed that age group had no effect on the presence of SEP, ramp teeth, fractured teeth and peripheral caries, but was related to all other abnormalities (Table 2). Not all abnormalities increased linearly with age; wave mouth and diastema were less common in the 6–10-year-old horses. According to the results of Cramer's V test, a high association was shown only with diastema and cupped out teeth (Table 2).

#### Discussion

The results of this study showed that 93.8% of horse heads examined had at least one dental abnormality. The most common abnormality found was SEP, in 68% of the heads. This is consistent with previous

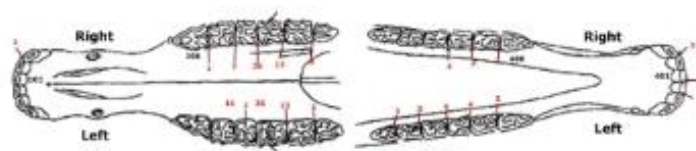


Figure 2. The results of periodontal pocket distribution between horse teeth from 89 heads. The number illustrates periodontal pockets found at each location with a total of 144 periodontal pockets.

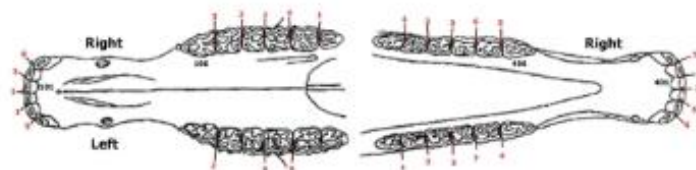


Figure 3. The results of diastema distribution between horse teeth from 52 heads. The number illustrates diastemata found at each location with a total of 107 diastemata.

## EQUINE

EQUINE

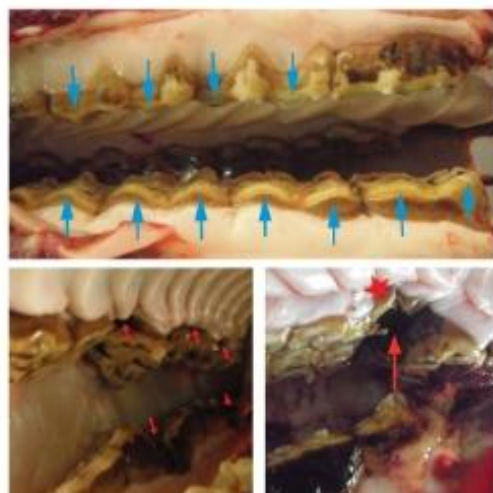


Figure 4. Equine dental abnormalities. (a) Exaggerated transverse ridges (blue arrow) in a 5-year-old horse, (b) wave mouth with severe periodontal disease (red arrows), (c) fracture in 109 with oronasal fistula (star and red arrows indicate the remnants of the fractured tooth, with oronasal fistula, respectively).

reports showing a prevalence ranging from 50% to nearly 100% of the population.<sup>5-7,14</sup> This wide range may be due to different definitions of SEP and examination criteria in each study. Because of its high prevalence, unless they are causing lacerations or other types of wounds to the surrounding soft tissue such as buccal mucosa and tongue, SEP could be considered as normal physiological changes

caused by routine eating and grinding behaviour, rather than an abnormality.<sup>7,14-16</sup> In our study, 32% of studied horses showed no evidence of SEP, but without a dental history it is unknown whether this was due to human intervention or natural wear. In addition, we were not able to assess facial muscle symmetry or ulceration of soft tissue because of the removal of facial muscles, cheeks and tongue during processing.

Tooth overgrowth was site specific; for example, hooks and ramps were most likely to be found on the rostral or caudal tooth of the cheek teeth arcades. Stepped teeth were most commonly found at the mandibular fourth premolar, which is the last of the cheek teeth to erupt. This may be associated with other pathologies such as retained deciduous fourth premolars and entrapment of the permanent tooth.

We found a higher prevalence of overshoot (parrot mouth) than in a Canadian abattoir survey (0.4%).<sup>7</sup> This might reflect differences in the definition of the malocclusion, or genetic or environmental factors in the different locations. However, one limitation of our study was that because most of the masticatory muscles were removed during processing, and the heads hung vertically, this allowed the mandible to fall forward. This may give the appearance of normal alignment in an actual overshoot incisor alignment, or conversely a normal appearance to an undershot incisor alignment.

Further investigation is required on the cause and relationship of overgrowth abnormalities and other malocclusions such as overshoot and undershot bite alignment. The correlation between malocclusion and the occurrence of periodontal disease in each age group is unclear, but risk factors are likely to be multifactorial.

Visible periodontal pockets were more likely to be found further caudally in the oral cavity such as between the fourth premolar (108, 208, 308, 408) and the first molar (109, 209, 309, 409) or other molars. This area has less movement of the tongue to shift food particles to the

Table 2. Prevalence of dental abnormalities in percentage of age groups with Pearson chi-square and Cramer's V test

	0-5 y (n = 134) n (%) <sup>a</sup>	6-10 y (n = 97) n (%) <sup>a</sup>	11-15 y (n = 120) n (%) <sup>a</sup>	>15 y (n = 49) n (%) <sup>a</sup>	Chi-square	P value	Cramer's V (P value)
SEP	84 (30.9)	73 (26.8)	83 (30.5)	32 (11.8)	4.325	0.228	0.104 (0.228)
Hook	38 (22.1)	44 (25.6)	71 (41.3)	19 (11.0)	25.094	<0.001	0.250 (<0.001)
Wave mouth	25 (24.5)	15 (14.7)	37 (36.6)	25 (24.5)	27.041	<0.001	0.260 (<0.001)
Periodontal pocket	21 (23.6)	16 (18.0)	32 (36.0)	20 (22.5)	16.326	0.001	0.202 (0.001)
ETR	27 (50.0)	19 (35.2)	7 (13.0)	1 (1.9)	19.702	<0.001	0.222 (<0.001)
Diastema	11 (21.2)	3 (5.8)	18 (34.6)	20 (38.5)	45.084	<0.001	0.336 (<0.001)
Ramp	10 (20.0)	15 (30.0)	17 (34.0)	8 (16.0)	4.849	0.183	0.110 (0.183)
Retained deciduous tooth	39 (88.6)	3 (6.8)	2 (4.5)	0 (0)	67.792	<0.001	0.412 (<0.001)
Stepped tooth	4 (11.8)	11 (32.4)	15 (44.1)	4 (11.8)	8.722	<0.001	0.148 (0.033)
Missing tooth	4 (19.0)	4 (19.0)	4 (19.0)	9 (42.9)	19.465	<0.001	0.221 (<0.001)
Fractured tooth	4 (25.0)	2 (12.5)	8 (50.0)	2 (12.5)	3.505	0.320	0.094 (0.320)
Mobile tooth	0 (0)	1 (9.1)	4 (36.4)	6 (54.5)	21.532	<0.001	0.232 (<0.001)
Cupped out tooth	5 (10.2)	4 (8.2)	9 (18.4)	31 (63.3)	136.159	<0.001	0.583 (<0.001)
Peripheral caries	0 (0)	1 (100)	0 (0)	0 (0)	3.132	0.372	0.088 (0.372)
One or more abnormalities observed	120 (89.6) <sup>b</sup>	91 (93.8) <sup>b</sup>	117 (97.5) <sup>b</sup>	47 (95.9) <sup>b</sup>	7.304	0.063	0.135 (0.063)

ETR, exaggerated transverse ridges; SEP, sharp enamel points; y, years

<sup>a</sup>Proportion of all studied groups i.e. the number of horses with hook at the age of 0-5 years was 38, which was 22.1% of all studied horses with hook.

<sup>b</sup>Proportion of each estimated age group i.e. the number of horses with at least one or more dental abnormalities at the age of 0-5 years was 120 horses or 89.6% of the horses aged 0-5 years.



Table 3: Association between age and dental abnormality compared to the reference group of horses aged 0–5 years

Dental abnormality	0–10 years		11–15 years		>15 years	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Hook	2.1 (1.2–3.6)	0.008	3.7 (2.2–6.2)	<0.001	1.6 (0.8–3.2)	0.180
Wave	0.8 (0.4–1.6)	0.527	1.9 (1.1–3.5)	0.025	4.5 (2.2–9.2)	<0.001
Stepped tooth	4.2 (1.3–13.5)	0.018	4.6 (1.5–14.4)	0.008	2.9 (0.7–12.0)	0.145
Periodontal pocket	1.1 (0.5–2.2)	0.866	2.0 (1.1–3.6)	0.033	3.7 (1.8–7.8)	<0.001
Diastema	0.4 (0.1–1.3)	0.122	2.0 (0.9–4.4)	0.094	7.7 (3.3–17.9)	<0.001
Missing tooth	1.4 (0.3–5.7)	0.642	1.1 (0.3–4.6)	0.874	7.3 (2.1–25.0)	0.002

CI, confidence interval; OR, odds ratio.

teeth for mastication, which may increase the chance of food particles becoming trapped interproximally.

The significance of pocket depth measurement was difficult to interpret because of the inability to determine total loss of attachment without using radiographic images of the teeth.<sup>20</sup> The second limitation of our study was assessing periodontal pocket depth by visual gross anatomic change alone, as radiation health risk precluded use of this modality in the abattoir work environment.

Diastemata were commonly found between incisors and cheek teeth of geriatric horses and this may be related to the wedge shape of geriatric teeth that creates spaces between adjacent teeth. The prevalence of diastema was significantly higher in >15-year-old horses (nearly 8-fold higher than in the 0–5 years age group). In young horses, tooth eruption creates spaces that allow feed particles to be trapped. Once eruption is completed, the teeth are tightly compressed at the occlusal surface. As the horse gets older, the wear of occlusal surfaces, especially of cheek teeth, causes tapering of the tooth and spaces called valve diastema.<sup>1,17</sup> These spaces allow accumulation of feed, which is a suitable environment for indigenous anaerobic gram-negative oral bacteria to grow. Subsequently, this will initiate periodontitis, including the loss of supporting structures such as periodontal ligament, the gum and alveolar bone.<sup>1,18,19</sup> However, it has been suggested that this periodontitis could be reversible, because of the continuous eruption of equine teeth.<sup>18</sup>

Our study showed that 11–15-year-old horses had at least one type of dental abnormality, similar to that reported in the Canadian abattoir study, although they did not detail the incidences of the various types of dental abnormalities with age.<sup>7</sup>

The abnormalities that involve eruption of the teeth, such as ETR and retained deciduous teeth, were more likely to be found in the 0–5-year age group. ETR may lead to more severe dental diseases such as diastema and periodontal disease because of the ridges forcing feed interproximally in the opposing arcade during mastication. A long-term investigation on ETR is needed before accepting ETR as a normal wear pattern for this age group.

Peripheral caries were found in only one 10-year-old horse between 109 and 110. This may be related to differences in nutrients in local feed, feeding practices or soil type in the Queensland region.<sup>8</sup>

In addition, cupped out teeth had the highest association with age among all observed abnormalities. This could related to normal

physiological changes of teeth structure where the infundibulae have lost their enamel layer and left only soft dentine, which will wear quicker, resulting in the oyster (cupped out) shape. This physiological change may need to be considered normal in the senior horse aged >16 years.

The third limitation of our study was that true age was difficult to determine because of the abattoir's work protocols. The method of age determination in horses by their dentition has been used for >100 years in horses aged <5 years using eruption of teeth.<sup>12</sup> Age estimation is less accurate as the age increases because of its reliance on occlusal wear patterns, but is based on changes of teeth structures in large populations of normal horses. Age grouping by estimated dental age remains the best approach for statistical analysis of age-related dental abnormalities.

There appears to be a lack of appreciation by horse owners of the signs of dental abnormalities.<sup>10</sup> Many studies have recommended annual dental examinations for horses aged >15 years, but our study shows that horses in all age groups had abnormalities that can be easily identified during a complete routine dental examination, with minimal equipment. The interval of dental examination and treatment will vary with individual horses because of different work discipline, diet, congenital or acquired abnormalities, practice experience and equipment used. We conclude that preventive dentistry introduced from a young age could correct the dental problems often missed by the horse owner and therefore prevent problems becoming clinically significant.

The results of this study will provide some predictions on what abnormalities are likely to be found in each age group. Further studies are required into the relationship between each dental abnormality and its clinical consequences.

Feral horse populations, which are on a natural diet and have no human intervention, will exhibit dental abnormalities associated with normal masticatory wear. No feral horses were included in this study. Future dental research on feral horses is likely to provide significant data on dental abnormalities.

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**Appendices to chapter 4**

The following appendices constitute data mentioned, but not published in Chapter 4: Isolation of bacteria associated with equine periodontal disease using culture dependent, and molecular methods.

**Table s8** The raw data of next generation sequencing using the Illumina platform from bacterial plaque sample collected from healthy and periodontitis affected horses (attached file)

**Table s9** Potentially novel bacterial species identified by 16S rRNA sequencing of bacterial isolate from 4 normal/gingivitis and 4 periodontitis samples; less than 98% identity

Species [% identity range]	Health/gingivitis	Periodontitis
	No of bacterial	No of bacterial
	isolate analysed	isolate analysed
	(% of total) n = 30	(% of total) n = 42
<i>Actinomyces suimastitidis</i> [95]		1
<i>Actinobacillus lignieresii</i> [97]	1	
<i>Actinomyces massiliensis</i> [95]		1
<i>Actinomyces urogenitalis</i> [96]		1
<i>Actinomyces viscocus</i> [91]		1
<i>Atopobium</i> spp. [85]	1	
<i>Campylobacter curvus</i> [90-91]	2	
<i>Campylobacter showae</i> [85]		1
<i>Campylobacter</i> spp. [96]	1	
<i>Campylobacter gracilis</i> [96]		1
<i>Citrobacter</i> spp. [88]	1	1
<i>Enterobacter</i> spp. [74]	1	1
<i>Eubacterium</i> spp. [86-91]		3
<i>Fusobacterium equinum</i> [96-97]	1	1
<i>Fusobacterium nucleatum</i> [96]	1	
<i>Fusobacterium</i> spp. [97]		2

<i>Lachnoanaerobaculum</i>		
<i>umeaense</i> [93-94]	1	1
<i>Olsenella profusa</i> [90]		2
<i>Olsenella</i> spp. [97]		1
<i>Parvimonas</i> spp. (canine oral) [97]	1	
<i>Peptostreptococcus anaerobius</i> [86]	1	
<i>Porphyromonas asaccharolytica</i> [87-90]	2	2
<i>Porphyromonas macacae</i> [ 91-94]	1	2
<i>Porphyromonas</i> spp. [93]		1
<i>Porphyromonas uenonis</i> [92-96]		2
<i>Prevotella dentasini</i> [78]	1	
<i>Prevotella jejuni</i> [95]		1
<i>Prevotella</i> spp. [91]	1	
<i>Slackia exigua</i> [94]	1	
<i>Staphylococcus epidermidis</i> [97]	1	1
<i>Streptococcus criceti</i> [96-97]	1	1
<i>Streptococcus</i> spp. [83-97]	1	3
Uncultured <i>actinobacterium</i> [97]		1
Uncultured bacterium [86-96]	7	7
<i>Veillonella atypica</i> [93]		2
<i>Veillonella</i> spp. [94-96]	2	1

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**Table s10** Bacterial species identified by 16S rRNA gene sequencing of isolates obtained from 4 normal periodontal tissue/gingivitis affected and 4 periodontitis affected horses (all at least 98% identity)

Species	Health/gingivitis No of bacterial isolate analysed (% of total) n = 23	Periodontitis No of bacterial isolate analysed (% of total) n = 20
<i>Actinobacillus equuli</i>	1	
<i>Actinomyces denticolens</i>	1	
<i>Actinomyces</i> spp.	2	
<i>Atopobium minutum</i>	1	
<i>Filifactor villosus</i> (canine oral)		1
<i>Fusobacterium</i> spp.	1	
<i>Lactobacillus alvi</i>		1
<i>Lactobacillus</i> spp.		1
<i>Paradardovia denticolens</i>		1
<i>Porphyromonas macacae</i>	1	
<i>Prevotella dentasini</i>	2	4
<i>Prevotella intermedia</i>	4	2
<i>Propionibacterium acnes</i>		1
<i>Propionibacterium</i> spp.	1	
<i>Propionibacterium</i> spp. (canine oral)		1

<i>Sharpea azabuensis</i>		1
<i>Staphylococcus aureus</i>	3	
<i>Staphylococcus epidermidis</i>		2
<i>Staphylococcus pasteurii</i>		1
<i>Staphylococcus</i> spp.	2	
<i>Streptococcus criceti</i>		2
<i>Streptococcus dentasini</i>	1	
Uncultured bacterium		2
Uncultured methanogenic archaeon	2	
Uncultured <i>Staphylococcus</i>	1	

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**Appendices to chapter 6**

The following appendices constitute data mentioned, but not published in Chapter 6: The effect of a chlorhexidine gluconate mouth rinse on equine oral microbiota determined by next-generation sequencing.

**Table s11** Result data from NGS of plaque samples of pre and post-trial of Chlorhexidine gluconate (attached file).

**Video 1** Hexarinse make it easy for horse dental care.wmv (attached file).